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THE UNIVERSITY OF ALBERTA

Effect of Zn^{2+} on Iron Assimilation in *Azotobacter vinelandii*

by

Marianne Huyer

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA

SPRING, 1989

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..... and I said,
"I am determined to be wise"
but this was beyond me.
Whatever wisdom may be,
it is far off and most profound —
who can discover it?
So I turned my mind to understand,
to investigate and to search out
wisdom and the scheme of
things .....

Ecclesiastes 7:23-25

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Effect of Zn^{2+} on Iron Assimilation in *Azotobacter vinelandii*" submitted by Marianne Huyer in partial fulfilment of the requirements for the degree of Master of Science.

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(Dr. M. A. Pickard)

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(External Examiner, Dr. A. Szalay)

Date: January 12, 1989

To my cat, Kermit

Primarily because he never asked me, "So, how is your thesis progressing?" or, "When are you going to Vancouver?". But also because his boundless curiosity about, and joy in, everything under the sun rarely failed to make me smile and furthermore served to remind me that there was more to life than my thesis.

ABSTRACT

The addition of 20 or 40 μM $ZnSO_4$ to iron-limited cultures of *Azotobacter vinelandii* caused early initiation of the rapid phase of production of the catechol siderophores and increased the amount of azotobactin. The total cellular iron level was not affected, suggesting that Zn^{2+} was not causing more severe, or earlier, iron limitation. Spectrophotometric examination of ion binding to the siderophores revealed that while the siderophores bound Zn^{2+}, only with azotochelin was iron unable to completely overcome any Zn^{2+}-induced changes in the absorption spectra. This appeared to rule out direct competition of Zn^{2+} with iron for siderophore binding. Also, Zn^{2+} did not appear to affect production of the putative ferrisiderophore receptor proteins.

The level of Fe^{2+} was significantly lowered in Zn^{2+}-grown cells, implying that Zn^{2+} inhibited the process of Fe^{3+} reduction. Since Zn^{2+} in either the pregrowth medium or the uptake solutions decreased both high- and low-affinity ^{55}Fe uptake, this suggested that Zn^{2+} inhibited an enzyme that was capable of reducing a variety of Fe^{3+}-chelates.

A ferric reductase was identified in soluble cell extracts. The specific activity of the enzyme was not affected by the concentration of iron in the growth medium but the enzyme was inhibited by Zn^{2+}. Purification of the enzyme by column chromatography resulted in the appearance, on an SDS-polyacrylamide gel, of two bands with molecular weights of 69,000 and 44,600. The enzyme utilized NADH as a reductant, required FMN as a cofactor, and was stimulated by Mg^{2+}. The apparent K_m values of the ferric reductase for NADH and Fe^{3+} were 15.8 and 10 μM, respectively. Analysis of the kinetic data using Lineweaver-Burke plots revealed a type of plot characteristic of a Ping Pong Bi Bi reaction mechanism. Kinetic analysis of the addition of Zn^{2+} to the enzyme indicated that Zn^{2+} was a hyperbolic (partial) mixed-type inhibitor. The enzyme was able to reduce an assortment

of iron chelates, including the ferrisiderophores, suggesting that its physiological role may be the dissociation of iron from the siderophores.

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ABBREVIATIONS

BSA	bovine serum albumin
2,3-DHBA	2,3-dihydroxybenzoic acid
EDTA	ethylenediaminetetraacetic acid
FAD	flavin adenine dinucleotide
Ferrozine	3-(2-pyridyl)-5,6-*bis*(4-phenylsulfonic acid)-1,2,4-triazine
FMN	flavin mononucleotide
NADH	ß-nicotinamide adenine dinucleotide, reduced form
NADPH	ß-nicotinamide adenine dinucleotide phosphate, reduced form
PMSF	phenylmethylsulfonylfluoride
sarcosyl	sodium lauroyl sarcosine
Tris	Tris(hydroxymethyl)aminomethane
Tris-HCl	Tris-hydrochloride buffer

INTRODUCTION

A brief description of the biology and chemistry of iron.

The presence of iron in many enzymes such as nitrogenase and ribonucleotide reductase, and in many cellular structures such as cytochromes and ferredoxins, make it an essential element for almost all forms of life (Lankford, 1972; Neilands, 1974). The extreme usefulness of this particular metal is related to its ability to exist in two stable redox states: Fe^{3+} and Fe^{2+}. The large range of reduction potential connecting these states allows iron to participate in electron transfer reactions in iron sulfur proteins and in cytochromes that have redox potentials between -490 mV and +385 mV (Lehninger, 1975). However, even though iron is the fourth most abundant metal on the earth's surface, it is not generally available for biological assimilation in aerobic environments (Neilands, 1974). This is due to the tendency of Fe^{2+} to spontaneously oxidize to Fe^{3+} which then undergoes hydrolysis and polymerization to form insoluble ferric hydroxides and oxyhydroxides at neutral pH. The result is that Fe^{3+} has a $K_{sol} \approx 10^{-38}$, which corresponds to a concentration of about 10^{-17} M – a level much too low for the sustenance of life (Spiro and Saltman, 1969). Since the initial development of life on earth occurred under anaerobic conditions, iron solubility was not a problem as Fe^{2+}, which is very soluble at neutral pH, was readily available to the different life forms. However, with the generation of O_2, the amount of iron that could be obtained easily decreased markedly, making it necessary for iron-requiring aerobic organisms to evolve a system that would enable them to acquire the iron that was vital to their existence (Neilands, 1972).

The characteristics and functions of siderophores.

The general microbial response to the situation of iron-limitation was the production of siderophores. These low molecular weight compounds (500 - 1000 daltons) have a high

affinity for Fe^{3+} (formation constant, $K_f > 10^{30}$) and are produced by most aerobic and facultative anaerobic microbes. There are a very small number of organisms which do not appear to produce siderophores: certain lactic acid bacteria (which seem not to require iron at all), *Legionella pneumophila*, *Neisseria* spp., and *Saccharomyces cerevisiae* (Neilands *et al.*, 1987). Heme probably serves as the major source of iron for the pathogens *Legionella* and *Neisseria*, and they must have unique iron-acquisitive capabilities enabling them to obtain iron from the intracellular environment in which they live. *S. cerevisiae* seems to be able to solubilize sufficient amounts of iron by acidification of the medium during growth such that siderophores are not required (Payne, 1987).

The term "siderophore" is Greek for "iron-bearer," and certainly the major role played by siderophores involves receptor-dependent, high-affinity iron transport. However, siderophore-type molecules are not restricted to this role. There are several potent antibiotics, the ferrimycins and the albomycins, which have siderophore-like structures (Hider, 1984); as well, siderophores can function as growth factors, an example being schizokinen from *Bacillus megaterium* (Byers *et al.*, 1967). Some siderophores can act as virulence factors: amonabactin, produced by *Aeromonas hydrophila*; aerobactin, produced by invasive strains of *Escherichia coli*; and anguibactin, produced by *Vibrio anguillarum*, are all elements of some consequence in the diseases caused by these organisms (Byers, 1987; Crosa, 1987).

The structure and chemistry of siderophores have been the object of much research, and this topic has been well reviewed (Hider, 1984; Lankford, 1972; Neilands, 1980, 1981b, 1984; Neilands and Leong, 1986; van der Helm *et al.*, 1987). Fe^{3+} has six octahedrally directed valence bonds and in dilute solutions is most effectively bound by structures that contain six coordinating atoms. These atoms are typically oxygens that are usually, but not invariably, supplied by catechol or hydroxamate functional groups. An exception is found in the siderophores parabactin, agrobactin, vibriobactin, and mycobactin in which the oxazoline nitrogen atoms appear to be involved in iron coordination (van der Helm *et al.*, 1987). Individually, siderophores exhibit a fair amount of structural variation, but when

examined as a group it appears that most of them can be classified as either hydroxamates or phenolates/catecholates (Fig. 1). The prototype hydroxamate siderophore, ferrichrome, was originally isolated from *Ustilago sphaerogena* and is a cyclic hexapeptide ferric trihydroxamate (Fig. 2A). The common and identifying characteristic of the catechol-type siderophores is the presence of mono- or dihydroxybenzoic acid functional groups. The prototypical example of this class is enterobactin (Fig. 2B) which although first isolated from *Salmonella typhimurium* and *E. coli* is not unique to these organisms as it has since been shown to be common to all enteric bacteria. Hydroxamates are produced by both prokaryotes and fungi and are thus a more common type of siderophore than the phenolates/catecholates which are only produced by bacteria. There are also a number of siderophores, including aerobactin and schizokinen, whose structures are based on citrate, and other compounds such as the plant phytosiderophores which, although lacking the "typical" functional groups, still seem to serve as siderophores.

Although there is a wide variety of siderophores, having different structures and characteristics, they all have in common the primary function of chelating Fe^{3+} for donation of iron to the organisms. Thus, they are integral to the ability of most organisms to survive in iron-limited environments.

Interaction of siderophores with metal ions other than Fe^{3+}.

Siderophore-mediated iron transport is a very efficient mechanism whereby cells obtain the iron essential for growth. However, in nature most organisms live in complex environments where there are many other metal ions, and although siderophores show a marked affinity for Fe^{3+}, these other ions could interact with the iron-binding ligands or could potentially have some other effect on iron assimilation.

Siderophores bind Fe^{3+} more readily than they do any other trivalent ion. Al^{3+}, with an ionic radius close to that of Fe^{3+}, has been used as a substitute for Fe^{3+} in NMR studies of

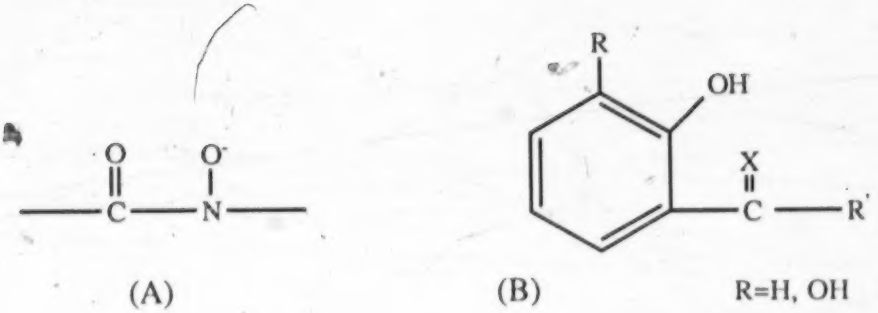

FIG. 1. Functional groups involved in the chelation of iron in (A) hydroxamate-type siderophores and in (B) phenolate/catecholate-type siderophores.

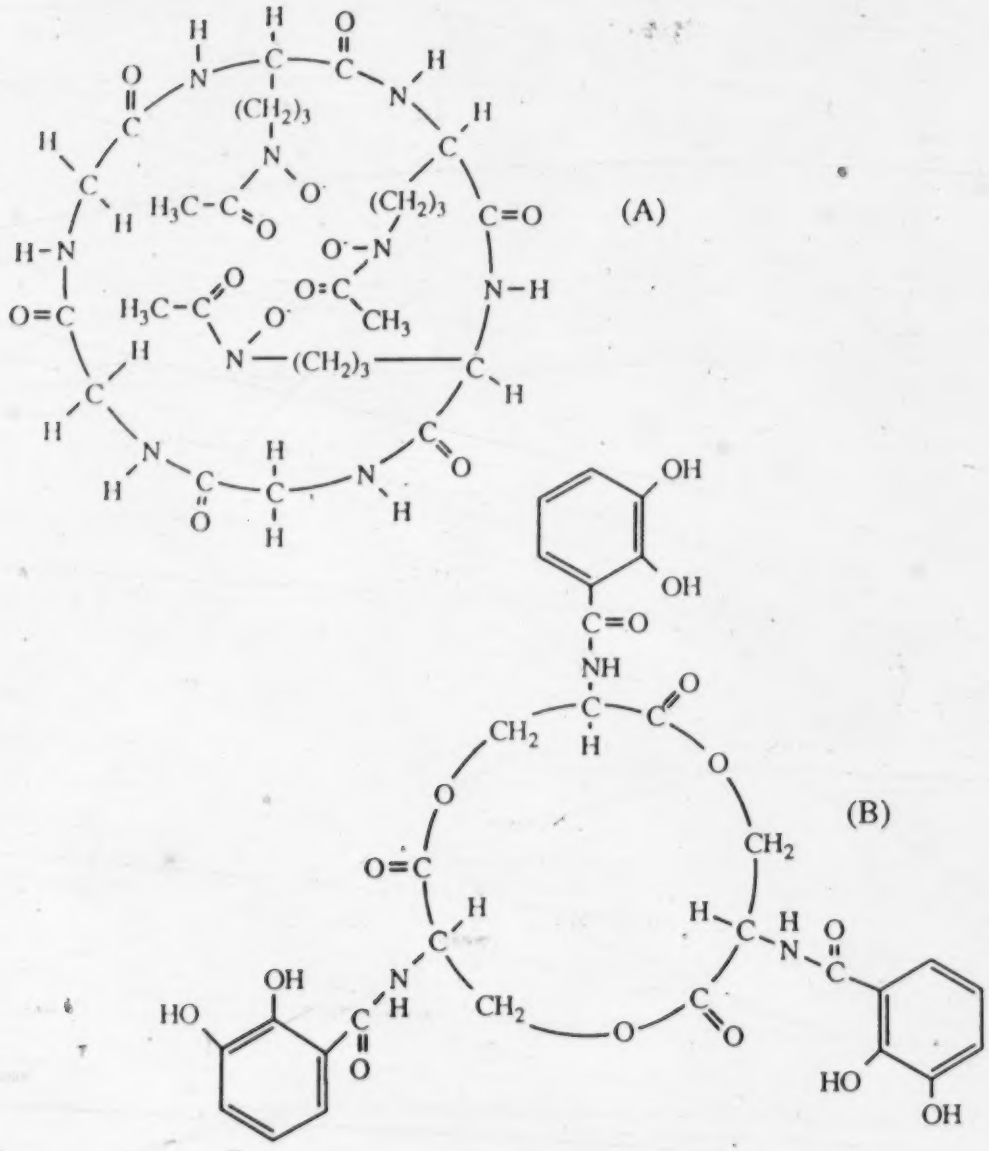

FIG. 2. Structures of the iron-free forms of (A) ferrichrome and (B) enterobactin. Both structures are redrawn from Crichton and Charloteaux-Wauters (1987).

ferrichrome and enterobactin, and although it does not bind as tightly to siderophores as does Fe^{3+}, its abundance in the biosphere might suggest that it could significantly compete with Fe^{3+} for siderophore binding (Hider, 1984; Neilands, 1981b, 1984). However, the inability of Al^{3+} to be reduced would inhibit its release from the siderophores, thus preventing this ion from substituting for iron in binding to proteins. Ga^{3+} binds quite well to siderophores and has been shown to compete effectively with iron in siderophore-mediated uptake in *U. sphaerogena* (Emery and Hoffer, 1980). But its extreme rarity in the biosphere prevents the possibility of its competition with iron from being a major problem. Cr^{3+}, although able to form stable complexes with siderophores, is kinetically inert, leading to difficulties in promoting the binding of this ion to the ligands.

Two metals which bind readily to siderophores are copper (Cu^{2+}) and molybdenum (Mo^{6+}) (Hider, 1984). Attachment of copper to the siderophore schizokinen produced by *Anabaena* spp. and *B. megaterium* results in decreased copper toxicity in *Anabaena* and increased toxicity in *B. megaterium* (Clarke *et al.*, 1987). Molybdenum, which exists in aqueous solution primarily as MoO_4^{2-}, has been shown to bind to several siderophores (Hider, 1984), including the *Azotobacter vinelandii* siderophores azotochelin and aminochelin (Page and von Tigerstrom, 1982, 1988). A similar anion, tungstate (WO_4^{2-}), acts in an analogous fashion. By binding to the siderophores, these ions have a potential to interfere directly with iron assimilation.

Effect of ions other than Fe^{3+} on siderophore production.

A fundamental point regarding siderophores is that their synthesis is promoted by cell growth under iron-limited conditions. But although iron plays the primary role in the regulation of siderophore synthesis, this by no means excludes other metal ions from being involved in this process.

The effects of other ions on siderophore production have been noted in a number of

organisms. Production of the lipid-soluble siderophore mycobactin by *Mycobacterium smegmatis* is dependent not only upon iron deficiency but also upon the presence of approximately 0.5 μM Mn^{2+} and Zn^{2+} (Ratledge and Hall, 1971). The negligible affinity that these metal ions have for mycobactin led to the suggestion that the increased siderophore levels seen in their presence is due to their action on some other component of the iron assimilation system, possibly one involving regulation of siderophore biosynthesis. When Al^{3+} was added to cultures of *B. megaterium* that contained a level of iron normally sufficient to repress schizokinen production, maximal production of the siderophore occurred, possibly due to direct competition of Al^{3+} with Fe^{3+} for entry into the cell (Davis *et al.*, 1971). With iron-sufficient cultures of *Neurospora crassa* grown in the presence of Co^{2+}, iron incorporation into heme was blocked, resulting in a marked increase in hydroxamate production (Padmanaban and Sarma, 1966). High levels of Co^{2+} in cultures of *U. sphaerogena* caused large quantities of ferrichrome to accumulate in the cells. This effect could be reversed by Zn^{2+}, leading to the suggestion that Zn^{2+} might be involved in iron utilization in this organism (Komai and Neilands, 1966). A variety of ions have been observed to affect the production of siderophores by *Pseudomonas fluorescens* (Baghdiantz, 1952; Chakrabarty and Roy, 1964). Of the divalent ions tested, it was found that Co^{2+} and Ni^{2+} caused siderophore production to decrease, albeit to a lesser extent than that seen with iron. Conversely, the addition of increasing amounts of Zn^{2+} and Mn^{2+} to the cultures caused increased siderophore production. As well, synthesis of the siderophores began earlier in the presence of Zn^{2+} than in its absence (Baghdiantz, 1952). The addition of low levels of Zn^{2+} (0.02 μg/mL) to iron-limited cultures of *B. megaterium* caused a significant increase in the yield of schizokinen (Byers *et al.*, 1967). And, although siderophore production was not specifically examined, it was noted that small amounts of Zn^{2+} were able to exaggerate the effects of extreme iron deficiency in *E. coli* (Ratledge and Winder, 1964).

An observation that the addition of Zn^{2+} to cultures of *Azotobacter vinelandii* caused an increase in siderophore production without severely affecting the cellular iron content was the impetus for the present study (M. Huyer and W. J. Page, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988. I60, p. 191). Although, as listed above, a number of early observations have been made on the effects of various ions on siderophore production and on iron metabolism, there has been little work done to determine the specific sites affected by these ions. Hopefully, an investigation into how other ions, in this case Zn^{2+}, give rise to the observed effects will lead to a better general understanding of the processes of iron assimilation and metabolism.

Biological roles of Zn^{2+}.

Zinc is rather unique amongst the biologically important transition metals in that it has only one oxidation state: Zn^{2+}. Although large quantities of Zn^{2+} are not essential for life, the normal metabolism and growth of all living systems does require small amounts of this metal ion. Zinc is a common constituent of soil and is found in widely differing amounts (typically from 10 - 300 ppm) depending on soil type and pH. Increasing acidity, finer soil texture, and higher quantities of organic matter increase the amount of available zinc. In acidic environments, zinc exists in a soluble form, while under basic conditions it is generally found in insoluble complexes (Shuman, 1980).

In man, Zn^{2+} deficiency can lead to small stature, anemia, low serum albumin, and retarded development of the reproductive system (Lehninger, 1982). In order to prevent these problems, man, as well as other higher life forms, synthesizes metallothioneins which function as metal-binding proteins that will also store Zn^{2+} (Cousins and Failla, 1980). Metallothioneins generally act to protect the cells against toxic metals and against unusually high levels of essential metals. However, because of their ability to store Zn^{2+} (and some other metals), it has been suggested that they may also play a role in normal zinc metabolism (Gallant and Cherian, 1986). In plants, the process of detoxification and

homeostasis of heavy metals has recently been found to be carried out by short peptides called phytochelatins (Grill *et al.*, 1985, 1987). However, except for yeasts, microorganisms do not appear to synthesize metallothionein-like proteins (Failla and Weinberg, 1980).

Besides metallothioneins, Zn^{2+} can be bound by ferritin, a multisubunit protein found in vertebrates, invertebrates, plants, fungi, and several bacterial species (in a form called bacterioferritin). Its major physiological role involves the storage, transport, and detoxification of iron (Crichton, 1984; Crichton and Charloteaux-Wauters, 1987; Harrison, 1979; Harrison *et al.*, 1980). However, ferritin is not necessarily specific for iron since it is also capable of binding a number of divalent ions including Zn^{2+}, Ni^{2+}, Hg^{2+}, Cd^{2+}, Co^{2+}, and Mg^{2+} (Macara *et al*, 1973). Zn^{2+} especially seems to bind well to ferritin and competes directly with Fe^{2+} for insertion into the binding sites (Zaman and Verwilghen, 1981).

The importance of Zn^{2+} in higher life forms also extends to microbial systems. Here, Zn^{2+} has been found to be essential to a number of major catalytic, structural, stabilizing, and regulatory functions (Failla, 1977; Failla and Weinberg, 1980). Thus, Zn^{2+} is involved in many cellular processes.

Catalytically, Zn^{2+} is found to be a component of many enzymes, known collectively as the zinc metalloenzymes. This is a very diverse group of enzymes found in both prokaryotes and eukaryotes, involving protein, carbohydrate, lipid, and nucleic acid metabolism (Failla and Weinberg, 1980).

E. coli alkaline phosphatase requires Zn^{2+} both for catalytic activity, with the ion being located at the active centre, and for structural stability, with the presence of Zn^{2+} ions between the two monomers lending stability to the dimer. There are a number of other enzymes, including *E. coli* aspartate transcarbamylase and *B. subtilis* amylase, which require Zn^{2+} strictly for stabilization of their quaternary structure. Zn^{2+} is also an important stabilizer of biological membranes (Chvapil, 1973; Failla, 1977). In this role, Zn^{2+} binds nonspecifically to the negatively charged head groups of membrane phospholipids, leading to a

decrease in electrostatic repulsion forces. In addition, binding of Zn^{2+} to free sulfhydryl residues of membrane proteins forms stable mercaptides which prevent the formation of free radical-induced disulfide linkages that would be detrimental to membrane structure and function (Failla, 1977).

In a combined structural and regulatory role, Zn^{2+}, in the form of zinc-fingers, has lately been found to be an important part of the DNA-binding domains of some regulatory proteins. Protein loops, rich in DNA-binding residues, which form around the Zn^{2+} ligands become correctly positioned to carry out sequence-specific binding to the DNA (Blumberg *et al.*, 1987; Nagai *et al.*, 1988). Zn^{2+} has also been shown to be involved in regulation of a variety of cellular functions including cytochrome formation and synthesis of δ-aminolevulinate dehydratase by *U. sphaerogena* (Failla and Weinberg, 1980).

Although Zn^{2+} is an essential element, it is generally required in such low amounts that the levels present as a contaminant in most media are sufficient. The difficulty of obtaining Zn^{2+}-free medium prevented early recognition of the importance of this metal ion; thus, Zn^{2+} has not received much attention until recently, and little is known about the mechanism by which it is obtained by most microorganisms. It has been suggested that transport of Zn^{2+} may occur through the magnesium transport system (Webb, 1970), or, depending on the organism, it may involve a specific transport system of its own (Silver and Lusk, 1987).

Iron uptake systems.

In contrast to the lack of information on Zn^{2+} uptake mechanisms is the relative wealth of information on iron uptake systems. The process of uptake of iron can be divided into two main systems. One, designated high-affinity, has three parts: the siderophores; the membrane receptors which participate in the transport of the iron into the cell; and a system that functions to release the iron from the ferrisiderophores. This high-affinity system is

quite specific in that most organisms produce their own unique siderophores (although some sharing between species exists) and that each siderophore has its own specific receptor protein. The second system is called the low-affinity uptake system. It is very non-specific and is primarily distinguished by its independence from the components of the high-affinity uptake system. Little is known about low-affinity iron uptake; in fact, the best evidence for its existence is that the growth of mutants lacking all components of the high-affinity system is not impaired provided that reasonably high levels of iron are present (Neilands, 1981, 1984).

The inability of the low-affinity iron uptake system to provide sufficient iron for growth in iron-limited media, or in media in which the iron is fairly strongly bound to chelators, illustrates the importance of having a high-affinity iron uptake system. The efficiency of the latter system means that adequate levels of iron are provided to the cells even when iron is not freely available (*i.e.*, the vast majority of cases). The siderophores are the vanguard of the uptake process: they chelate Fe^{3+} to form complexes from which the cell ultimately obtains iron. The second step in the uptake process involves the outer membrane receptor proteins. The importance of these proteins is a consequence of the cell wall structure of gram-negative bacteria: the outer membrane restricts all but quite small molecules (generally with molecular weights well below 500 daltons) from entering the cell. Porin proteins in the outer membrane form small water-filled pores which allow free entry of certain small, hydrophilic molecules (Nikaido, 1979). However, the ferrisiderophore complexes, although not extremely large, are for the most part too big to fit through these pores; thus, they require specific receptor proteins to assist in the transport of iron across the outer membrane (Neilands, 1982). Some of the ferrisiderophore receptors also render the cell more vulnerable to attack by certain bacteriophages; however, the requirement for iron is so great that this negative consequence of the uptake systems is far outweighed by the benefits that result from the ability to obtain iron (Neilands, 1979).

The components of iron uptake systems have been studied in a number of different

organisms, from gram-positive and gram-negative bacteria to fungi, and many clear and comprehensive reviews have been written on this subject (Byers and Arceneaux, 1977; Hider, 1984; Lankford, 1972; Neilands, 1980, 1981, 1982). Although differences do exist between organisms, the basic features of the uptake process seem to be fairly universal amongst aerobic, iron-requiring microorganisms.

Azotobacter vinelandii, the bacterium of interest in the present study, is a gram-negative nitrogen-fixing obligately aerobic organism commonly found in soil (Thompson and Skerman, 1979), and it has a fairly complex system for the uptake of iron. In order to meet its iron requirements, *A. vinelandii* produces three siderophores: a yellow-green fluorescent peptide called azotobactin (Demange *et al.*, 1986, 1987); azotochelin (2-*N*,6-*N*-di(2,3-dihydroxybenzoyl)-L-lysine) (Corbin and Bulen, 1969); and the recently described catecholamine siderophore, aminochelin (2,3-dihydroxybenzoylputrescine) (Page and von Tigerstrom, 1988) (Fig. 3). The synthesis of these compounds, as indeed of all siderophores, is derepressed under conditions of iron-limitation. In addition, 2,3-dihydroxybenzoic acid (2,3-DHBA) is produced constitutively and may function in low-affinity iron uptake (Page and Huyer, 1984). Production of the high-affinity iron chelators occurs in a sequential manner: azotochelin and aminochelin are synthesized first, while azotobactin is produced later and is only synthesized under conditions of severe iron-starvation (Page and Huyer, 1984). Once formed, the siderophores are secreted into the medium where they act to complex any free Fe^{3+} and, by virtue of their strong affinity for Fe^{3+}, remove iron from other less effective chelators. These three siderophores do not exhibit equal efficiency in transporting iron: the gradient of effectiveness is from azotobactin, which is the most effective, to azotochelin, and then to aminochelin (Knosp *et al.*, 1984; Page and von Tigerstrom, 1988). *A. vinelandii* also produces a number of iron-repressible outer membrane proteins. These have molecular weights of 93K, 85K, 81K, and 77K and presumably function as receptors for the ferrisiderophores (Page and Huyer, 1984; Page

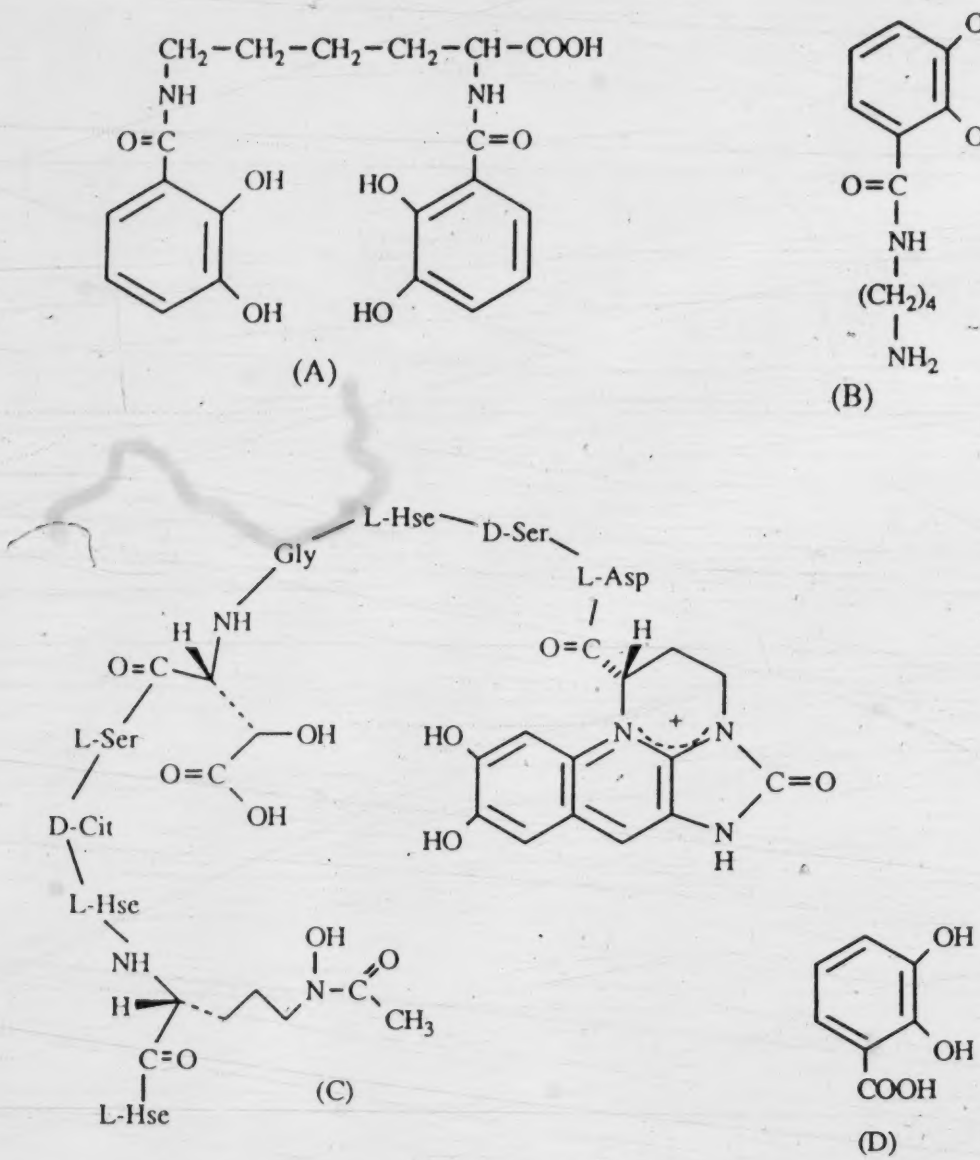

FIG. 3. Iron-binding compounds produced by *Azotobacter vinelandii*. (A) azotochelin (redrawn from Page and Huyer, 1984), (B) aminochelin (redrawn from Page and von Tigerstrom, 1988), (C) azotobactin (redrawn from Demange *et al.*, 1987), and (D) 2,3-DHBA.

and von Tigerstrom, 1982). It has been suggested that the 77K outer membrane protein may be specifically involved in azotobactin-mediated iron uptake (Page and Huyer, 1984; Page and Grant, 1988).

Iron uptake systems as a whole are quite complex, with many proteins being involved in the biosynthesis of the siderophores, in the transport of the ferrisiderophore complexes into the cells, and in the subsequent removal of the iron for utilization in various cellular processes. These systems, however, are not constitutively expressed. Although iron is an essential element, the existence of excess "free" iron in the cell can result in the production of hydroxyl radicals which are capable of inflicting a great deal of damage (Crichton and Charloteaux-Wauters, 1987). In order to prevent this, intracellular iron is generally found as a constituent of iron-requiring proteins or is stored in ferritin. Any free iron would probably be found in a transient pool of loosely coordinated Fe^{2+}, maintained in the reduced form by the strongly reducing environment of the interior of microorganisms (Bagg and Neilands, 1987). The appearances of this pool of free iron have been described as being "somewhat like the Loch Ness monster, only to disappear from view before its presence, or indeed its nature, can be confirmed" (Crichton, 1984). The potential toxicity of free iron means that regulation of iron uptake in response to the iron status of the cell is a vitally important process, and this necessitates a cellular ability to respond rapidly to changes in the levels of intracellular and extracellular iron.

Regulation of iron uptake.

There are several forms of iron which could potentially play a role in the regulation of iron uptake: siderophore-bound iron; iron in hemin and cytochromes; stored iron (*e.g.*, in bacterioferritin); and iron in the free, intracellular iron pool. Of these, siderophore-bound iron and heme do not appear to have any regulatory function. This conclusion is the result of a study that showed that *entA*$^+$ (for enterobactin production) strains had the same regulation of iron uptake as mutant *entA*$^-$ strains, and *hemA*$^-$*entA*$^-$ strains were also indistin-

guishable from *hemA*$^+$*entA*$^+$ strains in this respect (Klebba *et al.*, 1982). The role of bacterioferritin in iron metabolism is not well understood at the present time (Bagg and Neilands, 1987), and indeed, its presence has only been confirmed in a few species of microorganisms. Thus, it is difficult to know if, and if so how, iron in this form could be involved in the regulation of iron assimilation. However, the free iron in the cell is a likely candidate for functioning as an intracellular control (Williams, 1982). This pool would be depleted rapidly under iron-limited conditions which could quickly cause induction of the iron uptake systems.

The study of iron uptake systems shares common features with the studies of other systems in that certain areas have received a great deal of attention while other aspects have been largely ignored. In this regard, much is known about the various siderophores produced by different microorganisms, while the exact mechanisms by which their expression, and that of the iron uptake systems of which they are part, is regulated by the iron content of the medium are not well understood. Only in *E. coli*, an organism that has been well defined genetically, is much known about the system of regulation of iron uptake. This mechanism of regulation was first described in a mutant of *Salmonella typhimurium* which exhibited constitutive expression of several of the high-affinity iron uptake systems. The designation *fur*, for *f*erric *u*ptake *r*egulation, was given to this particular gene in *S. typhimurium* (Ernst *et al.*, 1978) and this has since been identified in *E. coli* (Hantke, 1981).

The *fur* system involves an iron-binding repressor, the Fur protein, which, when there is a sufficiently high intracellular level of iron, effectuates negative regulation of expression of the siderophore biosynthesis genes (Bagg and Neilands, 1987). This Fur protein, the product of the *fur* gene, is a divalent heavy-metal ion-activated DNA-binding protein. Although Fe^{2+} acts as the prime corepressor with Fur, other ions such as Mn^{2+}, Co^{2+}, Cu^{2+}, Cd^{2+}, and to a lesser extent Zn^{2+} are able to act in concert with Fur to promote Fur binding to iron-regulated promoter sequences (de Lorenzo *et al.*, 1987). Thus, Fur can be classi-

fied as a metal-activated protein, rather than as a metalloprotein, since the latter will not bind a variety of metal ions but instead has a very high affinity for one specific ion (Bagg and Neilands, 1987). As well, the Fur protein has been described as being prototypical of the metalloregulatory proteins because of its ability to cause metabolic changes in response to alterations in metal ion concentrations (de Lorenzo *et al.*, 1988).

The *fur* system for regulation of high-affinity iron uptake probably functions throughout the Enterobacteriaceae (Hantke, 1987), but, to date, it is entirely a matter of speculation as to whether this system or a closely related system operates in other organisms.

Regulation of the assimilation of iron by iron depends to a large extent on the ability of the organism to transfer extracellular iron to the intracellular pools. The siderophores function to chelate the iron and bring it to specific receptors so that it may be internalized. However, siderophores by design have an extremely high affinity for iron, and the simple process of bringing the iron to or into the cells does not guarantee that that iron is immediately available for use in other cellular processes. Therefore, microorganisms have had to develop special systems for the removal of iron from the ferrisiderophore complexes.

Release of iron from siderophores.

The separation of iron from siderophores is the final step in the process of iron assimilation and results in the iron being freed for use by the cell. After it is transported, the iron is incorporated into macromolecules at a much greater rate than that which could be obtained if nonenzymatic transfer of iron from the siderophores to another chelator was occurring. In fact, the process of detaching the iron from the siderophores is carried out by various enzyme systems which hydrolyze the ferrisiderophores and/or reduce the iron.

There are three main methods by which these enzyme systems free the iron from the ferrisiderophores (Bergeron, 1986; Crichton and Charloteaux-Wauters, 1987; Raymond and Carrano, 1979). The first, called the "taxi-cab mechanism," involves dissociation of the siderophore from its bound iron at the cell surface: the ferrisiderophore complex does

not enter the cell. By remaining extracellular, the siderophore is thus easily able to be reused by the organism. This mechanism is employed by *Rhodotorula pilimanae* as it obtains iron from its dihydroxamate siderophore, rhodotorulic acid (Carrano and Raymond, 1978). The siderophore forms a complex with the extracellular iron and donates the iron to the cell at the cell membrane, with release of the iron to a cell-bound iron chelating agent probably accomplished through the action of an iron reductase.

The second mechanism has been dubbed the "European" mechanism. This process, exemplified by ferrichrome, involves the transport of the intact ferrisiderophore complex into the cell followed by removal of the iron, presumably via an iron reductase, with subsequent re-excretion of the siderophore into the medium. The siderophore may or may not undergo some slight chemical modification during this process: ferrichrome is acetylated during iron release in *E. coli* but not in *U. sphaerogena*, while release of iron from aerobactin does not result in structural modification of the ligand (Crichton and Charloteaux-Wauters, 1987).

The third enzymatic mechanism, known as the "American approach" to iron assimilation, also involves transport of the ferrisiderophore complex into the cell, but the process of releasing the bound iron results in destruction of the ligand. This is utilized by the fusarinine-type siderophores produced by fungi such as *Fusarium roseum* (Emery, 1976) and by ferric enterobactin in its donation of iron to *E. coli* (O'Brien *et al.*, 1971: Porra *et al.*, 1972). Esterases in the cytoplasm of these organisms mediate the destruction of the ligands. Since ferric enterobactin has an extremely low redox potential, below the range of physiological reductants such as NADH and reduced flavin adenine dinucleotide, this implied that the ligand must first have to be modified to give a form with less affinity for Fe^{3+} before reduction of the iron could occur (Raymond and Carrano, 1979). However, since it has been shown that the ferrisiderophore complexes are not the preferred substrates for the esterases there is some confusion about the role of these enzymes in the release of iron from the siderophores. To further add to the unintelligibility of the whole matter is the

observation that enterobactin analogs that are not substrates for the ferric enterobactin esterase are able to provide useable iron to *E. coli* (Heidinger *et al.*, 1983). Recent studies suggest that protonation of ferric enterobactin, either in the low dielectric constant medium of the cytoplasm (Hider *et al.*, 1984) or in the acidic environment of the periplasm (Matzanke *et al.*, 1986), could result in a complex with a high enough redox potential for iron reduction to occur, but it is not known whether this is involved in *in vivo* iron release. As well, the exact role of the esterases needs to be elucidated further.

In spite of the lack of clarity about certain details of the process of iron release, double-label experiments have shown that in many cases the ferrisiderophore complex first enters the cell and then the iron is removed. Release of siderophore-bound iron through reduction of Fe^{3+} to Fe^{2+} is found in many different systems and is an effective mechanism because siderophores have markedly less affinity for Fe^{2+} than for Fe^{3+}. A number of different "siderophore reductases" have been described; however, there is presently some doubt as to the exact role that they play in the process of iron assimilation.

Ferric iron reductases.

The use of enzymatic reduction of iron as a mechanism for the release of iron from ferrisiderophores has been described as being both feasible and generally applicable (Arceneaux, 1983). The Fe^{2+} thus generated could be held by an acceptor molecule until needed or could be channelled directly into metabolic sequences.

Reductive mechanisms of iron assimilation involving ferrisiderophore reductases which catalyze Fe^{3+} reduction from ferrisiderophores to Fe^{2+} acceptors have been described in many species of microorganisms. Iron uptake in *U. sphaerogena* includes both the transport system for ferrichrome and a soluble ferrichrome reductase which carries out the subsequent iron release process (Emery, 1983; Straka and Emery, 1979). A ferrisiderophore reductase has also been described in *Neurospora crassa* (Ernst and Winkelmann, 1977), although it has been suggested that the use of EDTA in the assay for reduced iron

could have led to erroneous interpretation of the data since EDTA is capable of non-enzymatically reducing iron from ferrichrome (Straka and Emery, 1979). Other organisms which appear to possess an iron reductase include *Agrobacterium tumefaciens* (Lodge *et al.*, 1982), *Bacillus megaterium* (Arceneaux and Byers, 1980), *B. subtilis* (Gaines *et al.*, 1981; Lodge *et al.*, 1980), *Mycobacterium smegmatis* (Brown and Ratledge, 1975; McCready and Ratledge, 1979), *Pseudomonas aeruginosa* (Cox, 1980), and *Rhodopseudomonas sphaeroides* (Moody and Dailey, 1985). This list is by no means complete, and the apparent ubiquity of this method of iron reduction may result in iron reductases being described in most aerobic iron-requiring organisms.

These iron reductases are generally NAD(P)H:oxidoreductases and are dependent upon NADH or NADPH for reducing power. Most seem to require anaerobic conditions for activity, and some give higher activity in the presence of a flavin. The majority of these enzymes are soluble; however, an exception is the iron reductase of *Spirillum itersonii*, which has been described as being membrane-bound (Dailey and Lascelles, 1977).

Some iron reductases have been described as being multifunctional enzymes. In *B. subtilis*, a portion of the ferrisiderophore reductase activity was attributed to a flavin reductase found as part of a complex with chorismate synthase and dehydroquinate synthase of the aromatic biosynthetic pathway (Gaines *et al.*, 1981). As well, the finding of McCready and Ratledge (1979) that ferrimycobactin reductase activity exists in organisms other than mycobacteria led to the suggestion that the iron reductase activity could be associated with one or more ubiquitous proteins whose principal function might not be that of a ferrisiderophore reductase. Other examples include the NAD(P)H:flavin oxidoreductases of *Beneckea harveyi* and *E. coli*. The enzyme from *B. harveyi* in the presence of NADH and a flavin is able to reduce, and thus release, iron from ferritin (Jones *et al.*, 1978). The enzyme from *E. coli* acts as a ferric reductase in reducing the iron centre of ribonucleotide reductase, thus playing an important role in the radical-introducing reaction necessary to convert inactive ribonucleotide reductase to the active form (Fontecave *et al.*,

1987). The possible multifunctional characteristics of these iron reductases may explain why, in most cases, their activity is not affected by the iron status of the cells (Arceneaux, 1983).

Although most enzymes with iron reductase activity are described as siderophore reductases, they have not actually been shown to be involved in the process of iron transport (Emery, 1987). One argument against such a role is that these enzymes tend to lack substrate specificity. Thus, the iron reductase from *B. subtilis* will catalyze the reduction of iron from a number of ferrienterobactin analogs (Lodge *et al.*, 1980), and the enzyme from *U. sphaerogena* will reduce compounds from ferric citrate through to ferrichrome and ferrioxamine (Emery, 1987). Therefore, the physiological role of the iron reductases may be either one of simply being responsible for the maintenance of reduced iron in the cytoplasm (Emery, 1987) or a general one of reducing Fe^{3+} which is octahedrally coordinated to oxygen ligands, not just Fe^{3+} which is specifically bound to individual siderophores.

However, in spite of (or perhaps because of) this lack of specificity, these iron reductases could be very important to microbial iron assimilation. For this, it is necessary to envision an iron uptake system that consists of two separate parts (Emery, 1987). The first part of the system comprises the siderophores and the associated receptors and proteins. This system is very specific: different siderophores are produced by different organisms, and these organisms generally can only take up iron which is bound to their own siderophores.

The second portion of the system involves reductive removal of iron from the iron chelates at the cell surface rather than intracellularly. In this part transfer of iron across the cellular membrane(s) only occurs after reduction of Fe^{3+} to Fe^{2+}. For the ferrisiderophores that are transported intact into the cell, this kind of reductive mechanism would not be required for siderophore-mediated iron uptake. However, a general reductive system would allow the cells to acquire iron, albeit not very efficiently, from a wide variety of sources. The lack of specificity of this system, then, may be intentional, with iron

octahedrally coordinated to oxygen ligands being the substrate for reduction rather than the specific siderophores. Proteins bound to the cell membrane would presumably bind iron complexes in a non-specific fashion in order to allow this type of reductive activity to occur. Thus, the main purpose of the more specific siderophore system could be to enable the organisms to compete more effectively for iron in those situations where there is not enough available iron to satisfy the requirements of all the organisms in that milieu (Emery, 1987). Reduction, as mentioned above, is a plausible and likely mechanism for the removal of iron from ferrisiderophores, and most of the iron reductases so far described are capable of doing this. For siderophores which use the taxi-cab mechanism of iron uptake, this extracellular type of iron reduction could certainly assist the iron in entering the cell. For those ferrisiderophores which enter the cell intact, the cell might obtain the iron through the action of iron reductases in the periplasm or cytoplasm.

The indispensibility of iron to microorganisms implies that there must be multiple mechanisms for the assimilation of this metal ion: it is too necessary for most of life's processes for the cells to be totally dependent on a single uptake system. These mechanisms could range from the specific siderophore systems to the general reductive systems, and there is the potential of there being more, as yet undiscovered, systems (Emery, 1987).

In this light, it is interesting to note that nitrate reductase in plants, found both in leaves and in roots, appears to be able to reduce a wide variety of siderophores (Castignetti and Smarrelli, Jr., 1984). Previously, the presence of nitrate reductase in the roots was puzzling since the vast majority of nitrate reduction occurs in the leaves. However, the ability of this enzyme to reduce siderophore-bound iron as well suggests that its role in the roots may be to reduce iron in order to make this metal available for uptake. This, by necessity, is a non-specific reductive system, but it allows the plant to compete relatively successfully for the iron it needs for growth.

The acquisition of iron by most life forms is essential and therefore entails quite a complex process consisting of many systems containing many different components. Most

research to date has focussed on the siderophore system of iron uptake, but there is a wealth of knowledge to be obtained about other aspects of iron assimilation. Hopefully, this will eventually lead to a relatively clear picture of how this very insoluble but essential element is acquired by living organisms in quantities large enough to support life.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The capsule-negative strain UW of *Azotobacter vinelandii* OP was maintained on slants of iron-sufficient Burk medium (0.57 mM $CaSO_4$, 0.8 mM $MgSO_4$, 1.47 mM KH_2PO_4, 4.6 mM K_2HPO_4, 1 μM Na_2MoO_4, and 18 μM $FeSO_4$), pH 7.2, containing 1% glucose, 14.3 mM ammonium acetate, and 1.8% agar, incubated at 30°C. The inoculum for each study was pregrown on slants of Burk medium for 2-3 days. Cells were washed from the slants with iron-limited Burk medium without glucose and used to inoculate liquid cultures (200 mL per 500 mL Erlenmeyer flask) to an initial optical density at 620 nm of 0.08. Optical densitiy measurements were made with an LKB Novaspec spectrophotometer. Liquid Burk media did not contain $FeSO_4$ and was prepared using distilled, deionized water from a Milli-Q water purification system (Millipore Corp.) with glucose autoclaved separately. When required, 50 mg iron-containing minerals, ground to approximately 200 mesh, were added as a loose powder to media prior to autoclaving. Also when required, ferric citrate (filter-sterilized 5 mM stock solution) and $ZnSO_4$ (autoclaved 10 mM stock solution) were added just prior to inoculation. The minerals pyrite and Fe_3O_4 were obtained from Central Scientific Co. and Fisher Scientific Co. respectively. The other minerals used were obtained from the Department of Geology Museum (University of Alberta) or purchased from Wards Natural Science Establishment, Inc. In order to minimize iron contamination, all glassware was washed prior to use with 4 N HCl and 50 mM EDTA (pH 7). Liquid cultures were incubated for 20 hours at 30°C with shaking at 225 rpm either in a New Brunswick model G-76 gyratory water bath shaker or on a Lab-Line Orbit shaker.

For a growth curve, 20 mL cultures in 50 mL Erlenmeyer flasks were grown in triplicate in liquid Burk media containing 2 μM ferric citrate. Flasks corresponding to three separate but identical growth curve experiments were set up such that during a 13-h time

span, one set provided data on cell growth between 0 and 12.5 h, another set provided data for 10 to 19 h, and the third set provided data for 16 to 23.5 h. At approximately 2.5-h intervals, sets of three flasks were removed for analysis of protein, iron, and siderophore production as described below. The data from these overlapping curves were combined in order to construct a single growth curve.

Chemical Assays.

Protein was routinely determined by the method of Lowry *et al.* (1951) using BSA as a standard. Whole cells were predigested in 0.1 N NaOH at 80°C for 1 hour.

Iron determination using α,α'-bipyridyl (Osaki *et al.* 1971) was carried out on cell pellets that were washed with 10 mM trisodium citrate (pH 7) prior to being extracted with 7% perchloric acid overnight at room temperature and for at least 1 hour at 80°C. For cells that had been grown with minerals, the mineral was removed as described by Page and Huyer (1984) by overlaying the cell suspension on a 3 mL Percoll® (Pharmacia Fine Chemicals, Inc.) cushion. To determine the level of Fe^{2+} in cell extracts relative to the level of Fe^{3+}, the assay was carried out in the absence of added ascorbate.

"Free" iron in cytoplasmic and membrane fractions of sonicated cells was assessed using bleomycin-dependent degradation of DNA (Gutteridge *et al.* 1981). Modifications to the method included the use of deionized, distilled water from the Milli-Q water purification system in place of pyrogen-free water, and the use of acid-washed glassware in place of new plastic metal-ion-free disposable containers.

The amount of Zn^{2+} in samples was measured using a variation of a method employed in the determination of nitrilotriacetic acid (NTA) (Thompson and Duthie, 1968; Longman *et al.*, 1971). A 0.2 mL volume of standard Zn^{2+} (made up as 2 mM $ZnSO_4 \cdot 7H_2O$ in 8 mM Tris-HCl [pH 3]) was mixed with 2 mL of 0.5 mM zincon (2-carboxy-2'-hydroxy-5'-sulfoformazylbenzeme; Sigma Chemical Co.) in borate buffer (pH 9.2), and with 2 mL

water from the Milli-Q system, to provide a standard curve of nanomoles of Zn^{2+} *vs.* absorbance at 620 nm. Samples tested included cytoplasmic and membrane fractions obtained from high speed centrifugation (100,000 x *g*) of cell-free extracts.

Siderophore analysis.

After the cells were removed by centrifugation, the culture supernatant was acidified to pH 1.8 with HCl and scanned with a Perkin-Elmer Lambda 3 spectrophotometer. Levels of total catechols were estimated from the A_{310} (Page and Huyer, 1984) with purified 2,3-DHBA in iron-limited Burk medium (pH 1.8) as a standard. The A_{380} was used to estimate the levels of azotobactin in culture supernatants by using the known extinction coefficient (Fukasawa *et al.*, 1972). Extraction of the acidified culture supernatant with ethyl acetate (Corbin and Bulen, 1969) separated azotochelin and 2,3-DHBA from aminochelin and allowed the relative quantities of these catechols to be determined (Page and von Tigerstrom, 1988)

The *A. vinelandii* siderophores azotobactin, azotochelin, and aminochelin were purified as previously described (Knosp *et al.*, 1984; Corbin and Bulen, 1969; Page and von Tigerstrom, 1988). 2,3-DHBA was obtained from Sigma Chemical Co.

Interaction of Zn^{2+} with siderophores was measured by the method of Elad and Baker (1985). Cation solutions of 40 mM $ZnSO_4$, $FeCl_3$, and $Fe(NH_4)_2(SO_4)_2$ were prepared in acid-washed test tubes with water from the Milli-Q system. Purified siderophores in potassium phosphate buffer (4.6 mM K_2HPO_4, 1.47 mM KH_2PO_4 [pH 7.3]) were incubated with 0.1 mM $ZnSO_4$ for >1 h on ice in the dark. The absorption spectrum from 240 to 700 nm was compared with that for solutions incubated with 0.1 mM $FeCl_3$ or 0.1 mM $Fe(NH_4)_2(SO_4)_2$. Relative affinities for the metal ions were determined by measuring changes both in the absorption spectra of solutions to which Fe^{2+} or Fe^{3+} were added after

preincubation with $ZnSO_4$, and in the spectra of solutions to which Zn^{2+} was added after preincubation with $FeCl_3$ or $Fe(NH_4)_2(SO_4)_2$.

Preparation of outer membranes and electrophoresis.

Cells from 200 mL cultures were collected by centrifugation and suspended in 10 mL of 8 mM Tris-HCl (pH 7.8). The cells were broken by sonication (Branson cell disrupter; model 350) and treated with sodium lauroyl sarcosine (sarcosyl) in order to specifically isolate the outer membrane (Page and Huyer, 1984). Samples containing 20 µg protein were boiled for 5 min in sample buffer containing 2% sodium dodecyl sulfate (SDS), 8% (vol/vol) glycerol, and 5% ß-mercaptoethanol in 62.5 mM Tris-HCl (pH 6.8) before application to the gel. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970). Slab gels 1.5 mm thick were formed in a Bio-Rad model 520 electrophoresis cell and were run at 12-15 mA through the 5% stacking gel and at 24-30 mA through the 10% separating gel, at room temperature. The protein bands were stained with Coomassie blue in isopropanol and acetic acid as described by Fairbanks *et al.* (1971). The proteins used as molecular weight standards were phosphorylase A (94,000), bovine serum albumin (68,000), immunoglobulin G heavy chain (50,000), ovalbumin (43,000), immunoglobulin G light chain (23,500), and RNase (13,700).

^{55}Fe uptake assay conditions.

Iron-limited Burk medium without glucose used in the preparation of uptake medium was autoclaved and allowed to stand for one week; then it was filtered through a 0.8 µm-pore-size membrane filter (Millipore Corp.) the day before use to remove insoluble salts. Glucose was added to 0.5% and trisodium citrate was added to 10 mM. Cells were incubated for 16.5 h as described above and harvested by centrifugation. Cultures grown in the

presence of minerals were held static for 10 min prior to harvesting to allow the minerals to settle out (Page and Grant, 1988). Cell pellets were washed twice with uptake medium at 4°C, resuspended to an optical density at 620 nm of approximately 9.0 in uptake medium, and held on ice prior to use. Culture supernatants used in ^{55}Fe uptake assays were filtered through a 0.45 μm-pore-size Millipore filter and supplemented with 0.5% glucose and 10 mM trisodium citrate. Prior to use in ^{55}Fe uptake assays, all purified siderophores were diluted to desired levels with uptake medium. $ZnSO_4$ was added to selected uptake solutions to a final concentration of 40 μM. The $^{55}FeCl_3$ (1 mCi/mL in 0.1 N HCl; Amersham Corp.) was mixed with unlabeled $FeCl_3$ (in 10 mM trisodium citrate) to give a stock solution of 179 μM Fe^{3+} and 20 μCi ^{55}Fe/mL. This radioactive stock was diluted into the various uptake solutions to give a final assay concentration of 2.7 μM Fe^{3+} and 0.3 μCi ^{55}Fe/mL. ^{55}Fe uptake assays were performed as previously described (Knosp *et al.*, 1984). Radioactivity was determined with a Beckman model LS 3801 liquid scintillation counter.

Enzymatic assays.

The aerobic reduction of Fe^{3+} to Fe^{2+} was measured by trapping the product as an Fe^{2+}-ferrozine complex using a slightly modified version of the assay described by Dailey and Lascelles (1977). The 2.5 mL assay mixture consisted of 0.4 mM NADH, 0.16 mM ferric citrate, 1 mM $MgCl_2$, and 1 μM FMN, in 10 mM Tris-HCl (pH 7.6), incubated together in the dark for 10 min prior to the addition of ferrozine to 0.16 mM. The reaction was started with the addition of enzyme and the increase in absorbance at 562 nm was followed spectrophotometrically at room temperature with either a Perkin-Elmer Lambda 3 UV/VIS spectrophotometer or a Pye Unicam SP8-400 UV/VIS spectrophotometer. Activity was defined as the nanomoles of Fe^{2+} formed per minute per mg of protein.

Location of ferric reductase activity in nondenaturing discontinuous polyacrylamide gels was carried out essentially as described by Moody and Dailey (1983). The gels were run at 15 mA through the 5% stacking gel and 30 mA through the 10% separating gel at room temperature. Use of the activity stain resulted in the appearance of pink-purple bands and spots in the gel after 20 to 30 min.

In the determination of the location of the ferric reductase activity in the cell fractions, citrate synthase was used as a marker enzyme for the cytoplasm. Citrate synthase activity was measured in extracts of cells fractionated by the gentle methods described below. The activity was determined by the method recommended by Boehringer Mannheim Co. as the decrease in A_{232} in a 3 mL assay mixture (at 25°C) containing 0.1 M Tris-HCl (pH 8.0), 50 µL oxaloacetate (1.32 mg/mL), 50 µL acetyl-CoA (10 mg/mL), and various amounts of the different cell extracts.

Gentle methods of cell fractionation.

(A) Lysozyme treatment: Cells harvested by centrifugation at 10,400 x *g* were washed with water from the Milli-Q water purification system (at 4°C) and suspended in 33 mM Tris-HCl (pH 8.0) containing sodium citrate, sucrose, and lysozyme (Hughes *et al.*, 1971). After a 10 min incubation at room temperature, Mg^{2+} was added and the "protoplasts" were collected by centrifugation at 400 x *g*. The protoplasts were sonicated following resuspension in Tris-buffered sucrose. The soluble fraction (presumably cytoplasm) was separated from the membrane fraction by centrifugation at 100,000 x *g*.

(B) Cold-osmotic shock: Harvested cells were washed twice with 40 mM Tris-HCl (pH 7.8) and resuspended in 160 mL Tris buffer containing 25% sucrose and 1 mM EDTA. After 10 min at room temperature the suspension was centrifuged at 10,400 x *g* and the pellet was rapidly dispersed in 160 mL of ice-cold 0.5 mM $MgCl_2$ (Hughes *et al.*, 1971). After a final centrifugation, concentration of the supernatants to a final volume of

~10 mL was carried out on ice using an Amicon PM-10 ultrafiltration membrane (Amicon Corp.).

(C) Glycerol shock: Harvested cells were washed twice with 25 mM Tris-HCl (pH 7.4). The pellets were then suspended in 8 mL Tris buffer containing 4 M glycerol, held at room temperature for 30 min, and centrifuged at 12,000 x *g*. The resulting pellet was vigorously resuspended in 8 mL of Tris buffer containing 10 µg DNase/mL and centrifuged at 27,000 x *g* (Eady, 1980). To isolate the cell membranes, the pellets were resuspended in Tris buffer containing 10% sucrose and 0.1 mg lysozyme/mL, left at room temperature for 30 min, and then placed on ice for 1 h. The chilled suspension was centrifuged at 1935 x *g*. Membrane purification was achieved through the use of sucrose step gradients described by Page and von Tigerstrom (1982).

Purification of ferric reductase.

Nine litres of Burk medium containing 5 µM ferric citrate as the iron source (glucose and ammonium acetate autoclaved separately) in a Magnaferm fermenter (New Brunswick Scientific Co., Inc.) were inoculated to an initial optical density at 620 nm of 0.2 with one litre of cell culture obtained from 200 mL liquid cultures prepared as described above. Cultures were grown at 30°C with 8 L oxygen per minute at an agitation speed of 400 rpm and were harvested after 20 h using a Pellicon cassette system (Millipore Corp.) equipped with a 0.5 µm-pore-size membrane filter. The resulting cell slurry was centrifuged in order to pellet the cells. The cells were then washed with 10 mM Tris-HCl (pH 7.6) and suspended in 10 mM Tris buffer to a final volume of approximately 120 mL. The cells were broken in the presence of DNase I and RNase with a French pressure cell at 20,000 lb/in^2, 4°C, followed by treatment with lysozyme (Page and von Tigerstrom, 1982). The cell extract was centrifuged at 100,000 x *g* for 90 min and the soluble fraction used as the source of ferric reductase.

Column chromatography was carried out at 4°C essentially according to the method of

Moody and Dailey (1985) with slight modifications described below. Approximately 1/4 of the soluble material was applied to the initial DEAE-Sephacel® column. Buffer A, used for the DEAE-Sephacel columns and the Phenyl Sepharose® CL-4B column, consisted of 10 mM Tris-HCl (pH 7.6) and 10% (wt/vol) glycerol. The ferric reductase activity was eluted from the initial DEAE-Sephacel column (2.5 x 28 cm) with 0.35 M KCl in buffer A. Pooled fractions were concentrated by ultrafiltration with an Amicon PM-10 ultrafiltration membrane and reapplied to the DEAE-Sephacel column which had been equilibrated with 0.1 M KCl in buffer A. The ferric reductase was eluted with a 600 mL KCl gradient (0.15 to 0.5 M) in buffer A. Pooled, concentrated fractions, adjusted to 0.6 M KCl, were applied to a Phenyl Sepharose CL-4B column (1.5 x 14 cm) which had been equilibrated with 0.6 M KCl in buffer A. The column was eluted with 0.6 M KCl in buffer A followed by buffer A alone. The ferric reductase eluted in the presence of the salt. Pooled fractions were concentrated to approximately 2 mL and applied to a Sepharose® 4B column (1.5 x 30 cm) which had been equilibrated with buffer B (10 mM Tris-HCl (pH 7.6), 10% (wt/vol) glycerol, 0.5 M KCl, and 7 μM FMN). The column was eluted with buffer B. All column material was obtained from Pharmacia Fine Chemicals, Inc.

RESULTS

Siderophore production in the presence of Zn^{2+}.

A. vinelandii was grown in the presence of iron-containing minerals in order to effect hyperproduction or repression of all or part of the siderophore system, relative to the iron-starved control (Page and Huyer, 1984; Table 1). The minerals used comprise three different groups: group 1, containing the minerals glauconite and pyrite, repressed azotobactin synthesis but permitted low levels of catechol production; group 2, containing the minerals olivine and hematite, partially repressed azotobactin synthesis and fully derepressed catechol production; and group 3, containing the mineral micaceous hematite, caused hyperprodution of both types of siderophores. Depending on the iron source, cells were either iron-limited (siderophore production partially derepressed) or iron-starved (siderophore production fully derepressed). In all instances, except when cells were grown with glauconite, increased production of azotobactin was concomitant with the presence of Zn^{2+} (Fig. 4). This increase was frequently very spectacular: cultures grown with 40 μM Zn^{2+} in the presence of pyrite, olivine, or hematite exhibited a significant increase in the level of azotobactin over that seen with the corresponding Zn^{2+}-free cultures (Table 1). This was particularly dramatic in the case of pyrite since azotobactin was not produced in the absence of Zn^{2+} but was produced in its presence (Table 1 and Fig. 4). Production of catechols also increased in the presence of Zn^{2+} with the amounts of the catechol-type siderophores synthesized under these conditions increasing in equal proportions: the ratio of azotochelin to aminochelin to 2,3-DHBA remained the same regardless of the presence of Zn^{2+} and of the iron source used (data not shown). The only exception to this was when the iron source was micaceous hematite, where the production of catechols appeared to be at a maximal level and could not be increased further. Similar results were obtained with other minerals tested (data not shown) including the group 2 mineral Fe_3O_4 and the group 3 mineral

TABLE 1. Effect of Zn^{2+} on siderophore production[a]

Mineral	Ideal Formula	Zn^{2+} (μM)	Cellular iron (μg of Fe/mg of protein)[b]	Siderophore Production (μmol/g protein)		Siderophore derepression[d]	
				Catechol[c]	Azotobactin	Catechol	Azotobactin
None		0	0.082	765	24.9	1.00	1.00
		20	0.137	850	43.6	1.11	1.75
		40	0.094	726	30.5	0.95	1.22
Glauconite	See footnote *e*	0	0.731	287	0	0.38	0
		20	0.782	360	0	0.47	0
		40	0.603	450	0	0.59	0
Pyrite	FeS_2	0	0.498	463	0	0.61	0
		20	0.449	541	12.8	0.71	0.51
		40	0.485	742	23.7	0.97	0.95
Olivine	$(Mg,Fe)_2SiO_4$	0	0.295	728	1.5	0.95	0.06
		20	0.232	826	17.3	1.08	0.69
		40	0.233	930	20.4	1.22	0.82
Hematite	Fe_2O_3	0	0.238	718	5.7	0.94	0.23
		20	0.203	784	110	1.02	4.43
		40	0.186	940	146	1.23	5.88
Micaceous hematite	Unknown	0	0.249	1015	32.3	1.33	1.30
		20	0.258	1001	45.5	1.31	1.83
		40	0.183	991	50.6	1.30	2.03

[a] All values are means of at least three separate experiments.
[b] Cell-associated iron per milliliter of culture relative to cellular protein per millilitre of culture.
[c] Mixture of 2,3-DHBA, azotochelin, and aminochelin.
[d] Siderophore derepression relative to the Fe-starved, Zn^{2+}-free control.
[e] Glauconite ideal formula: $K_2(Mg,Fe)_2Al_6(Si_4O_{10})_3(OH)_{12}$.

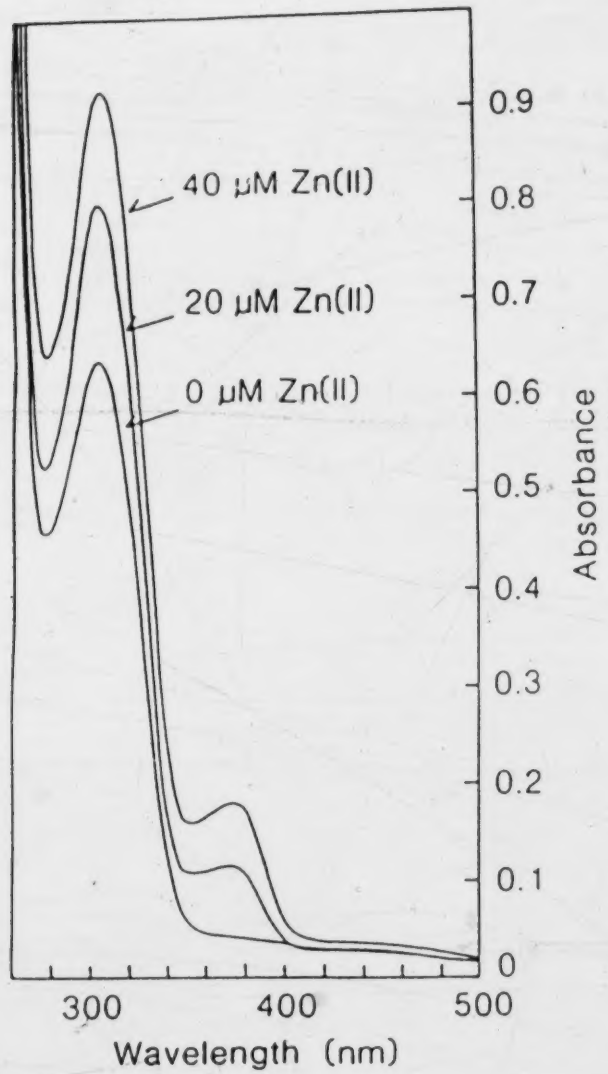

FIG. 4. Absorption spectra of *A. vinelandii* siderophores in culture supernatants. Cultures were grown with pyrite in the presence of 0, 20, and 40 μM $ZnSO_4$. Quantity of the catechol-type siderophores (comprising azotochelin, aminochelin, and 2,3-DHBA) indicated by A_{305}, and of azotobactin by A_{380}.

illite-bearing shale ($2K_2O \cdot 3(Mg,Fe)O \cdot 8(Al,Fe)_2O_3 \cdot 24SiO_2 \cdot 12H_2O$) (Page and Huyer, 1984).

Growth pattern in the presence of Zn^{2+}.

Iron-limited cultures grown in the presence and absence of Zn^{2+} showed identical growth characteristics: a 2 to 3 h lag phase was followed by a 17 to 18 h log phase which led into the stationary phase of growth (data not shown). After 20 h of incubation, iron-starved cultures had a protein content of approximately 230 µg protein per mL, while cultures grown in the presence of iron had a protein content of 300 - 500 µg/mL, depending on the amount of available iron present. The addition of 20 - 40 µM Zn^{2+} to iron-starved cultures did not significantly affect the protein content, while a similar addition of Zn^{2+} to iron-limited cultures caused a decrease in protein content of between 5 and 20%. Up to 200 µM Zn^{2+} could be added without further decreasing cell growth and protein content (data not shown). This indicated that cell viability and growth were not deleteriously affected by even relatively high concentrations of Zn^{2+}.

The addition of Zn^{2+} to iron-limited cultures appeared to cause slight variations in the cellular iron content (Table 1); however, these changes were not significant. This suggested that Zn^{2+} was not affecting the ability of the siderophores to solubilize iron from the mineral sources, a conclusion supported by the observation that the addition of Zn^{2+} to cultures containing a soluble iron source (Fig. 5) resulted in the same pattern of increased siderophore production as that seen with the insoluble mineral iron sources (Table 1). Most importantly, the presence of Zn^{2+} did not cause the cellular iron level to drop to the low level characteristic of iron-starved cells. This indicated that it was unlikely that the increased siderophore production was a result of a Zn^{2+}-induced iron starvation condition.

During the growth of a culture containing 2 µM ferric citrate, the phase of rapid produc-

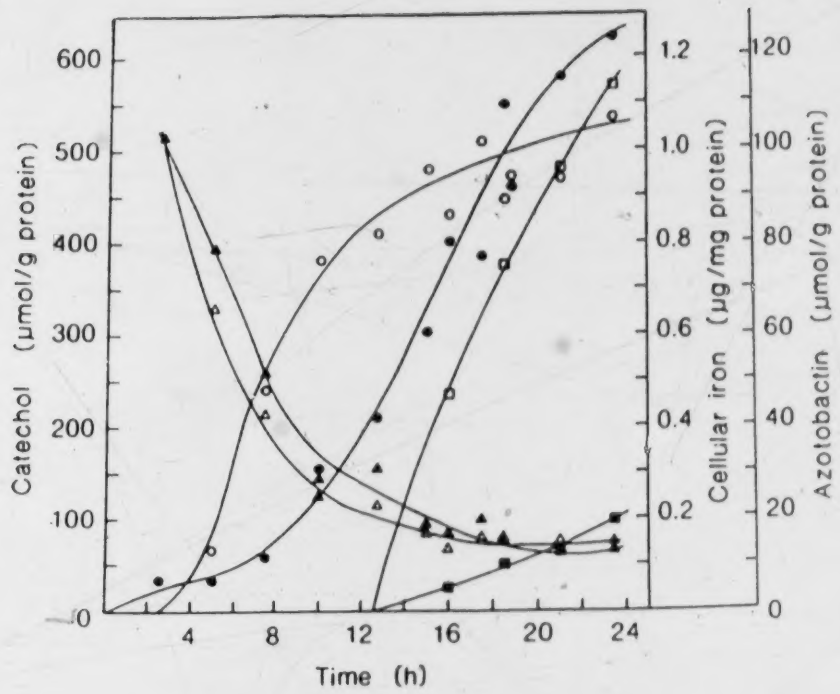

FIG. 5. Effect of Zn^{2+} on siderophore production over a 24 h time span. Cultures were grown in the presence (open symbols) and absence (solid symbols) of 40 µM $ZnSO_4$. Reported activities include cellular iron (△ , ▲), catechol production (○ , ●), and azotobactin production (□ , ■).

tion of the catechol-type siderophores began much earlier in the presence of 40 μM $ZnSO_4$ than in the absence of Zn^{2+}, although by the time the cultures reached the end of the exponential growth phase the total level of catechols in the two cultures was comparable (Fig. 5). Similarly, azotobactin was hyperproduced in the presence of Zn^{2+}. However, the amount of cellular iron remained essentially parallel with the control culture over the entire growth period. The early production of catechol siderophores and azotobactin occurred at cellular iron/protein values that were not characteristic of iron-starved cells. Therefore, zinc did not appear to bring about a condition of more severe iron limitation or of earlier iron limitation, both of which were possible explanations for the increased production of siderophores.

Effect of Zn^{2+} on outer membrane proteins.

Iron-repressible outer membrane proteins, which on SDS-polyacrylamide gels exhibited molecular weights of 93,000, 85,000, 81,000, and 77,000 (Fig. 6A and B) are presumed to be ferrisiderophore receptors and have been previously described for *A. vinelandii* (Page and von Tigerstrom, 1982; Page and Huyer, 1984; Page and Grant, 1988). The addition of $ZnSO_4$ to iron-limited and iron-starved cultures did not result in a decrease in the production of the iron-repressible proteins (Fig. 6A and B). In fact, cultures grown in the presence of Zn^{2+} appeared to produce greater amounts of these iron-repressible proteins, a result consistent with the concomitant increase in the production of the siderophores since the biosynthetic genes for the siderophores and the receptors are derepressed simultaneously (Neilands, 1982). The normal expression of these proteins in *A. vinelandii* cultures containing Zn^{2+} suggested that the increased levels of siderophore production was not due to Zn^{2+}-mediated repression of putative receptors.

SDS-polyacrylamide gel electrophoresis of outer membranes from cultures grown in the presence of 0 to 50 μM $ZnSO_4$ revealed that correspondingly increasing amounts of the 60,000-molecular weight surface protein (60K surface protein) (Bingle *et al.*, 1984)

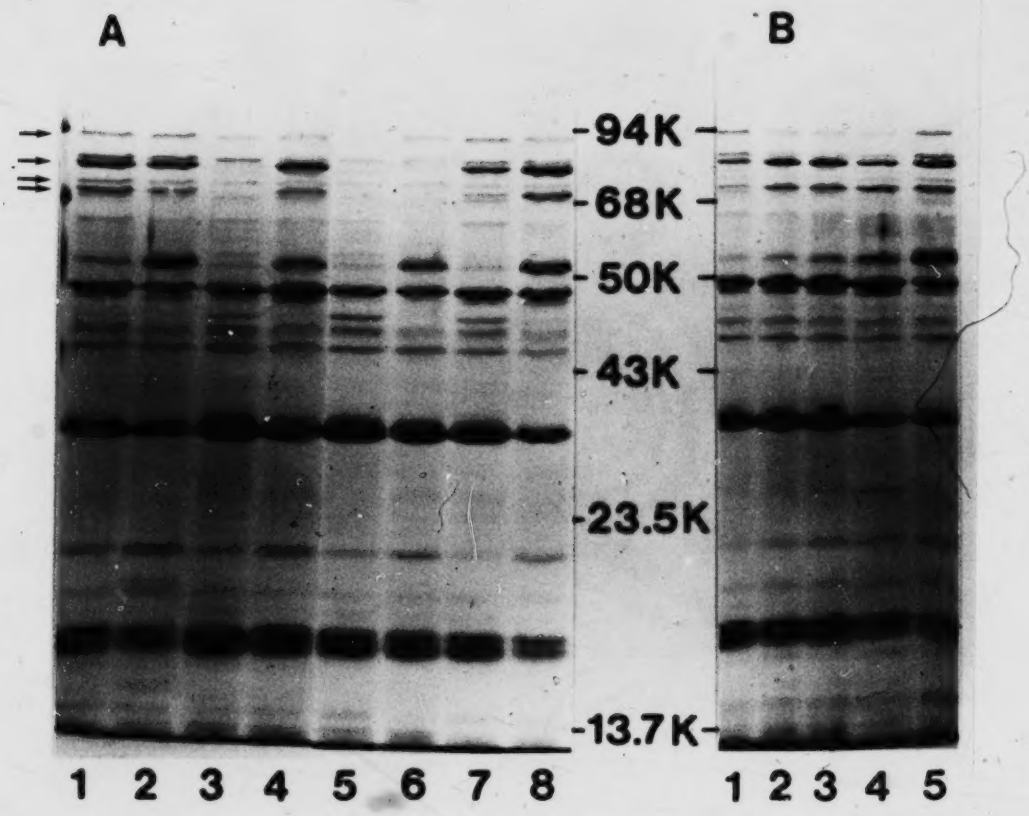

FIG. 6. SDS-polyacrylamide gel electrophoresis of outer membranes prepared by sarcosyl extraction. (A) *A. vinelandii* was grown in the absence (lanes 1, 3, 5, and 7) and presence (lanes 2, 4, 6, and 8) of 40 μM $ZnSO_4$ in iron-limited Burk medium containing no added iron (lanes 1 and 2), or the minerals Fe_3O_4 (lanes 3 and 4), glauconite (lanes 5 and 6), and olivine (lanes 7 and 8). (B) Growth of *A. vinelandii* with olivine in the presence of increasing concentrations of $ZnSO_4$: 0 μM (lane 1), 5 μM (lane 2), 10 μM (lane 3), 20 μM (lane 4), and 50 μM Zn^{2+} (lane 5). Arrows indicate position of iron-repressible proteins.

remained associated with the outer membrane (Fig. 6B). This appeared to be analogous to the results obtained during growth of *A. vinelandii* in the presence of excess iron, wherein the iron was thought to form a complex with the surface layer protein (Page and Huyer, 1984), thus promoting a continued association of the surface layer with the outer membrane during sarcosyl extraction. The association of the zinc ions with the cell surface was consistent with the role of this layer in cation binding (Doran *et al.*, 1987). Zn^{2+} also appeared to affect the presence of a protein with a molecular weight of approximately 45,000 which was not iron-repressible; however, this effect was not always observed (Fig. 6A and B).

Ion binding to siderophores.

Another possible explanation for the observed increased production of siderophores during growth in the presence of Zn^{2+} was that the zinc ions were binding competitively with iron to the siderophores, thus necessitating increased siderophore production to obtain equivalent levels of iron. Characteristic changes in the wavelength of maximum absorbance of the siderophores were seen upon the addition of Fe^{3+}, Fe^{2+}, and Zn^{2+} (Table 2). However, in all instances, except with azotochelin (Fig. 7A and B), Zn^{2+} appeared unable to successfully compete with iron, since the addition of Fe^{3+} or Fe^{2+} to siderophore solutions that had been preincubated with Zn^{2+} resulted in absorption spectra characteristic of those seen with Fe^{3+} or Fe^{2+} alone (data not shown).

The addition of Fe^{3+} or Fe^{2+} to the catechol-type siderophores gave rise to a new absorbance maximum at approximately 550 nm. The absorbance corresponding to this blue-violet adduct was not affected by the order in which Zn^{2+} and Fe^{2+} were added to the siderophore solutions. However, when Fe^{3+} was added to azotochelin that had been preincubated with Zn^{2+}, the A_{540} was at a much lower level that that reached with Fe^{3+} alone (Fig. 7A). There also appeared to be a similar, if less dramatic, effect when Fe^{3+} was added to aminochelin that had been preincubated with Zn^{2+}.

TABLE 2. Wavelength of maximum absorbance of purified siderophores of *Azotobacter vinelandii* in the presence of Fe^{3+}, Fe^{2+}, and Zn^{2+}

	Absorbance maximum (nm)[b]			
Added ion[a]	Azotobactin	Azotochelin	Aminochelin	2,3-DHBA
none	407	310	323	303
Fe^{3+}	407	320	329	303
Fe^{2+}	405	328	327	302
Zn^{2+}	420	335	323	301
$Fe^{3+} + Zn^{2+}$	406	330	328	300
$Fe^{2+} + Zn^{2+}$	406	327	325	302

[a] Each ion was added to a concentration of 0.1 mM. The order of addition of ions to the siderophores did not affect the results.

[b] Absorbance maxima determined from spectrophotometric scans of siderophore solutions.

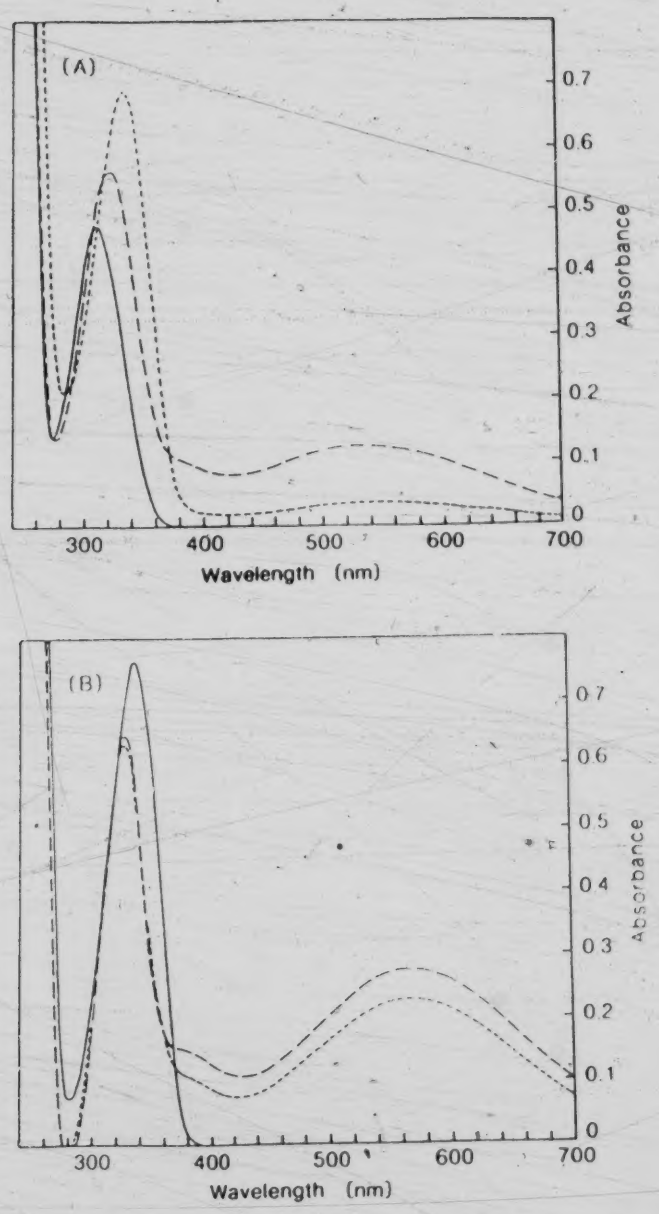

FIG. 7. Absorption spectra of azotochelin. The spectra presented are of purified siderophore alone (solid line, A), and after the addition of 0.1 mM Fe^{3+} (long dashes, A), Fe^{2+} (long dashes, B), Zn^{2+} (solid line, B), Fe^{3+} after preincubation with Zn^{2+} (short dashes, A), and Fe^{2+} after preincubation with Zn^{2+} (short dashes, B).

The results presented above suggested that although the siderophores appeared to bind Zn^{2+}, they preferentially bound iron. Of all the siderophores, azotochelin seemed to have the greatest affinity for Zn^{2+}, as evidenced by the inability of Fe^{3+} to completely overcome the Zn^{2+}-induced changes in the absorption spectrum (Fig. 7A and B). However, *A. vinelandii* normally produces three siderophores in vast excess of the amount needed for iron uptake (Page and von Tigerstrom, 1988), and it seemed unlikely that Zn^{2+} bound to azotochelin could so severely affect iron uptake as to necessitate further hyperproduction of the siderophores.

^{55}Fe uptake assays

^{55}Fe uptake experiments were performed with iron-starved cells as well as with cells grown in the presence of olivine. The use of mineral-grown cells introduced the possibility that the assay might be affected by release of unlabeled iron from the cell surface or by contaminating mineral. However, as previously shown (Page and Grant, 1988), washing the cells twice with uptake medium containing 10 mM trisodium citrate was sufficient to prevent any significant solubilization of unlabeled iron from the cell surface during the assay.

Cells grown with olivine had a higher level of ^{55}Fe uptake than iron-starved cells. This was most evident in low-affinity uptake with 2,3-DHBA or buffer and in high-affinity uptake with aminochelin, the least efficient of the three siderophores in transporting iron (Page and von Tigerstrom, 1988). In these instances, olivine-grown cells took up 60 to 80% more iron than the iron-starved cells (data not shown). In high-affinity uptake with azotobactin or azotochelin, olivine-grown cells acquired up to 25% more iron than the iron-starved cells. These results suggested that the presence of a small amount of iron during cell growth (enough to partially repress siderophore production but not repress production of the iron-repressible high molecular weight outer membrane proteins) may have relieved some of the stress imposed by iron-starvation conditions, thus increasing the

metabolic activity of the cells and allowing a greater rate of iron uptake.

The addition of 40 μM Zn^{2+} to uptake solutions caused an approximately 60% decrease in ^{55}Fe uptake by *A. vinelandii* that had been pregrown with olivine (Table 3). Cells grown in the presence of Zn^{2+} exhibited lower ^{55}Fe uptake than cells grown without Zn^{2+} and in most instances showed a minor decrease in uptake when Zn^{2+} also was added to the uptake solutions. The addition of Zn^{2+} to the uptake solutions alone or to the pregrowth medium resulted in a maximum inhibition of uptake of approximately 50 to 60%. Since the levels of uptake of ^{55}Fe were similar in cells pregrown with Zn^{2+} to that in Zn^{2+}-free cells to which Zn^{2+} was added in the uptake solutions, this suggested that Zn^{2+} was not in direct competition with iron for uptake into the cells (Table 3). Inasmuch as the stability constants of Zn^{2+} with citrate are comparable to those of Fe^{2+} and Fe^{3+} with citrate (Perrin, 1979; Sillén and Martell, 1964) and washing the cells with citrate prior to the ^{55}Fe uptake assay removes cell-bound iron (Page and Grant, 1988), there should be little cell-bound Zn^{2+} to interfere with the assay. Interestingly, the increased amount of the 60K surface protein that remained associated with the outer membrane during sarcosyl extraction of Zn^{2+}-grown cells (Fig. 6A and B) was not seen with Zn^{2+}-grown cells that were washed with citrate prior to sarcosyl extraction (data not shown).

An exception to this general depressive effect of Zn^{2+} on ^{55}Fe uptake appeared to be in the azotobactin-mediated ^{55}Fe uptake by the olivine-grown cells. As with the other siderophores, the addition of Zn^{2+} to the uptake solution containing azotobactin reduced ^{55}Fe uptake considerably in Zn^{2+}-free cells (Table 3). However, the amount of ^{55}Fe taken up by Zn^{2+}-grown cells was similar to that acquired by Zn^{2+}-free cells (Table 3). It seemed, therefore, that Zn^{2+} had an immediate, depressive effect on iron uptake, but when *A. vinelandii* was grown in the presence of Zn^{2+}, azotobactin-mediated iron uptake was able to adapt to the presence of this metal ion and thus could overcome the repressive effects.

Similar results were obtained in the examination of ^{55}Fe uptake by iron-starved cells

TABLE 3. Effect of 40 μM Zn^{2+} on ^{55}Fe uptake by *A. vinelandii* pregrown with olivine

Uptake solution[a]	Pregrowth with 40 μM Zn^{2+}	^{55}Fe uptake[b] (ng of ^{55}Fe/g of protein)	% of maximum ^{55}Fe uptake[c]
Azotobactin	–	26.5	100
	+	23.0	86
Azotobactin + Zn^{2+}	–	10.2	38
	+	18.3	68
Azotochelin	–	17.5	100
	+	7.30	42
Azotochelin + Zn^{2+}	–	7.46	43
	+	7.11	41
Aminochelin	–	23.0	100
	+	14.0	61
Aminochelin + Zn^{2+}	–	9.20	40
	+	11.0	48
2,3-DHBA	–	16.5	100
	+	8.13	49
2,3-DHBA + Zn^{2+}	–	6.08	37
	+	6.27	38
Buffer	–	14.9	100
	+	5.48	37
Buffer + Zn^{2+}	–	5.13	34
	+	5.83	39

a When required, $ZnSO_4$ was added to 40 μM to uptake solutions kept on ice in the dark.

b ^{55}Fe uptake values have been corrected for ^{55}Fe bound to the cells in the same solutions incubated on ice for 10 min. All values are means of a minimum of three separate experiments.

c Maximum ^{55}Fe uptake arbitrarily chosen as that acquired by Zn^{2+}-free cells with each Zn^{2+}-free uptake solution.

with the exception that the depression of uptake caused by Zn^{2+} was less than that observed in olivine-grown cells (data not shown). The addition of Zn^{2+} to either the pregrowth medium or to the uptake solutions caused a 20 to 40% decrease in ^{55}Fe uptake by iron-starved cells.

Status of cellular iron.

It was conceivable that Zn^{2+} could have indirectly caused hyperproduction of the *A. vinelandii* siderophores by affecting the form of cellular iron or the distribution of iron within the cell without affecting the amount of iron accumulated. This could potentially have an affect on the regulation of siderophore production.

A method involving bleomycin (an antibiotic that will only degrade DNA in the presence of Fe^{2+} ions), developed for the determination of "free" iron (*i.e.*, iron which is not protein-bound) in biological fluids such as synovial fluid, cerebrospinal fluid, and pleural-exudate fluid (Gutteridge *et al.*, 1981) was used in an effort to determine the "free" iron content of the *A. vinelandii* cytoplasm. The rate of DNA degradation, resulting in the formation of a product that reacts with thiobarbituric acid to form a chromogen, is used to assess the quantity of "free" iron. The prepared standard curve (Fig. 8) was reasonably linear between 0 and 5 nmol Fe^{2+}; however, the A_{532} values recorded were much lower than those obtained by Gutteridge *et al.* (1981). Most test assays, containing 5 µL volumes of soluble cell extract, gave A_{532} values within the range of the standard curve. As expected, the amount of iron with which the cultures were grown was reflected in the amount of "free" iron detected by the bleomycin assay (Table 4). The presence of Zn^{2+} during growth of the cultures did not appear to affect the amount of "free" iron in the soluble fraction of the cells (Table 4). However, a test for the possible interference of Zn^{2+} with the bleomycin assay revealed that the addition of as little as 0.5 nmol of Zn^{2+} to the assay mixture caused the A_{532} to decrease by approximately 15%. This effect was magnified as the

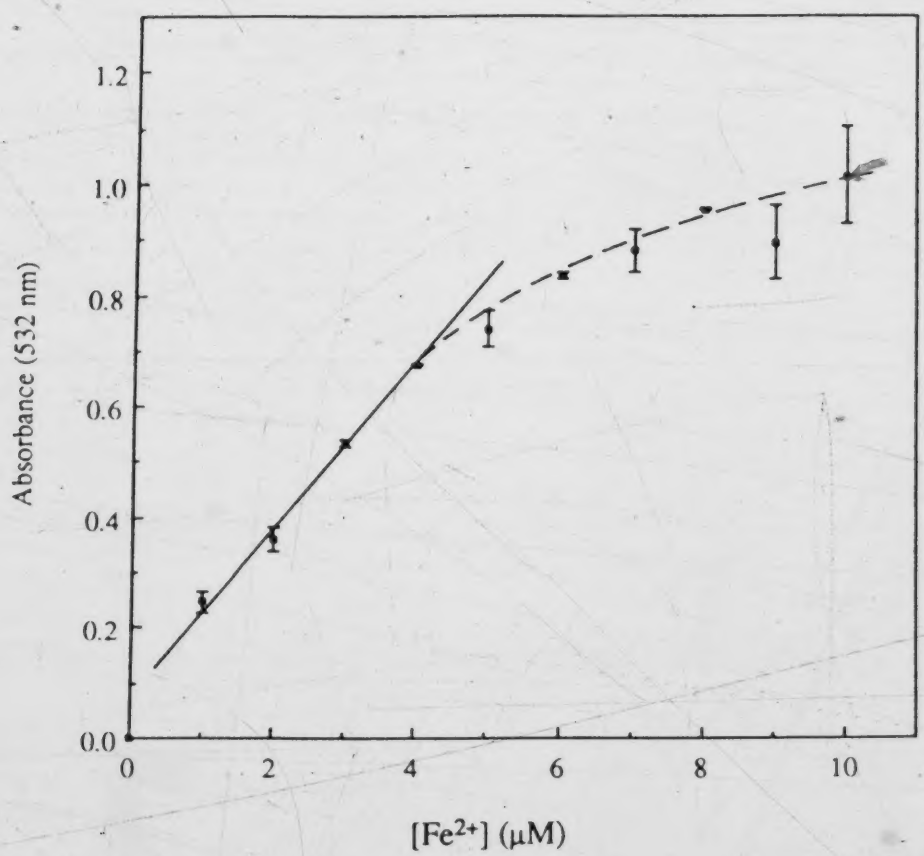

FIG. 8. Standard curve for the bleomycin assay. Curve indicates the formation of the thiobarbituric acid-reactive product from the Fe^{2+}-dependent degradation of DNA by bleomycin in the presence of various amounts of Fe^{2+}. Dashes indicate nonlinear portion of plot. The bars represent the range of values obtained from a minimum of three separate experiments.

TABLE 4. Determination of "free" iron in soluble cell extracts of *A. vinelandii*

Growth conditions		A_{532}[a]	"Free" iron (nmol of Fe^{2+}/mL of sample)
Fe (μM)	Zn (μM)		
0	0	0.496	560
1	0	0.785	986
1	40	0.784	980
2.5	0	1.25	–[b]
2.5	20	0.934	–
2.5	40	1.22	–

[a] A_{532} values determined from bleomycin test assays containing 5 μL of soluble cell extract.
[b] –, not calculated.

amount of added Zn^{2+} was increased.

A zincon assay was used in an attempt to determine if the level of Zn^{2+} within the cells was at a high enough level to significantly interfere with the bleomycin assay. Zn^{2+} reacts with zincon to produce a coloured complex with a maximum absorbance at 620 nm. However, while this assay provided a standard curve that was linear up to and beyond 200 nmol Zn^{2+}, it was inadequate for the detection of less than 10 nmol of Zn^{2+}. Furthermore, when Fe^{2+} (obtained from a stock solution of $FeSO_4$ in HCl) was substituted for Zn^{2+} in the zincon assay, the A_{620} increased concomitantly with the iron. This resulted in a linear plot with a slope of 2.62 A_{620} per μmol Fe^{2+}, 20% of that obtained with Zn^{2+} alone. The addition of this acid iron solution to the assay did not cause the pH to drop to a level at which the accuracy of the assay would be affected. Fe^{3+} (from a stock solution of $FeSO_4$ in distilled water) reacted to a lesser extent with the zincon and gave a plot with a slope of 0.74 A_{620} per μmol Fe^{3+}, 5.8% of that obtained with Zn^{2+}. When zinc and iron were combined, the colour of the assay changed from brown to purple and quickly faded.

The mutual interference of zinc and iron detailed above, and the problem of detecting very low levels of Zn^{2+} unfortunately made it impossible to determine with any degree of accuracy whether or not the level of "free" iron within the cells was altered by the presence of Zn^{2+} during cell growth.

Another possibility was that cell growth in the presence of Zn^{2+} could result in an alteration in the relative amounts of Fe^{2+} and Fe^{3+} compared with that seen in Zn^{2+}-free cells. This was examined through an analysis of the iron content of the membrane and soluble cell fractions as well as of the complete cell-free extracts from cultures grown in the presence and absence of 40 μM Zn^{2+} (Tables 5 and 6). The large standard deviation obtained for many of the samples (Table 5) was a consequence of the difficulty of removing all traces of mineral from the cell pellets and of the inability of the α,α'-bipyridyl assay to

TABLE 5. Fe^{3+} and Fe^{2+} content of fractions of *A. vinelandii* cells grown in the presence and absence of 40 μM Zn^{2+}

Iron source	Zn^{2+} (μM)	Amount of Fe^{3+} and Fe^{2+} in cell fractions[a] (μg of Fe/g of protein)					
		Cell-free extract		Soluble fraction		Membrane fraction	
		Fe^{3+}	Fe^{2+}	Fe^{3+}	Fe^{2+}	Fe^{3+}	Fe^{2+}
None	0	92.9 (26.7)[b]	41.7 (16.4)	61.4 (21.8)	43.6 (18.9)	62.6 (23.7)	62.5 (49.2)
	40	87.3 (16.8)	43.7 (20.3)	61.1 (23.2)	51.2 (11.7)	32.2 (0.1)	31.2 (0.2)
Glauconite	0	309 (67.9)	572 (25.0)	71.0 (6.9)	316 (36.7)	217 (55.6)	164 (19.1)
	40	213 (14.0)	328 (128)	105 (42.8)	188 (8.7)	242 (95.4)	114 (73.2)
Olivine	0	162 (20.0)	154 (34.9)	36.7 (6.0)	202 (32.1)	183 (73.6)	77.4 (31.1)
	40	156 (28.0)	58.4 (31.8)	52.1 (4.9)	63.0 (17.8)	104 (10.9)	35.2 (18.5)

[a] All values are means of a minimum of three separate experiments.
[b] Values in brackets denote standard deviation.

accurately measure very low levels of iron. The presence of Zn^{2+} during cell growth did not appear to affect the amount of Fe^{3+} in the cell-free extracts (Table 5). However, there was a distinct difference in the level of Fe^{2+} in the cell-free extracts and in the soluble fractions of cells that were grown with glauconite or olivine in the presence or absence of Zn^{2+} (Table 5).

Ferric reductase activity in extracts of *A. vinelandii*.

The iron content of the growth medium appeared to have a slight effect on ferric reductase activity. Cells grown with more than 1.25 μM iron exhibited a marginally higher level of enzyme activity than cells grown with less iron (Table 6). However, this may be a reflection of the potentially higher metabolic activity of iron-sufficient cells in relation to that of iron-limited cells rather than a direct dependence of the enzyme activity on the level of iron.

Cells that were grown in the presence of 40 μM Zn^{2+} showed a general decrease in the activity of the ferric reductase. The degree of enzyme inhibition seemed to be related to the iron concentration of the growth medium in that the ferric reductase activity of iron-sufficient cells was affected by Zn^{2+} to a much greater extent than that of iron-limited cells (Table 6). However, the amount of Zn^{2+} acquired by the cells also appeared to be related to the amount of iron present during cell growth since the cellular zinc levels increased as the degree of iron-limitation decreased (Table 6). Therefore, the apparent relationship between iron and the degree of enzyme inhibition may be fortuitous, with the significant factor actually being the concentration of zinc within the cells. Interestingly, an examination of the relative levels of Fe^{3+} and Fe^{2+} in the soluble cell extracts revealed that the cells in which the enzyme was inhibited by Zn^{2+} had a much lower amount of Fe^{2+} than the cells in which the enzyme was not so inhibited (Table 6). At all of the tested levels of iron, the presence of Zn^{2+} caused the expected increase in siderophore synthesis (Table 6). Similar data were

TABLE 6. Effect of iron and zinc on ferric reductase activity and siderophore production

Growth conditions		Siderophore production[a]		Soluble iron (μg of Fe/g of protein)		Ferric reductase activity[c]	Cellular zinc[d] (μg of Zn/ g of protein)
Fe (μM)	Zn (μM)	Azotobactin	Catechol[b]	Fe^{3+}	Fe^{2+}		
0.5	0	++	+++	48.8	47.2	4.96	24.4
	40	+++	+++	53.6	53.4	3.70	508
1.25	0	+	+++	90.3	18.7	5.25	23.5
	40	++	+++	119	12.5	1.34	1517
2.5	0	–	++	100	82.7	6.67	27.7
	40	+	+++	106	23.6	1.16	1652
3.75	0	–	+	113	169	8.11	23.0
	40	+/–	++	173	48.5	0.73	N.A.[e]
5.0	0	–	+/–	137	159	7.06	13.6
	40	–	+	157	49.0	0.56	2036
10.0	0	–	–	167	298	7.48	N.A.
	40	–	+/–	201	98.5	0.53	N.A.

[a] Indicates relative levels of siderophores: +++, maximal; ++, slightly below maximal; +, low; +/–, barely detectable; and –, not detectable.

[b] Mixture of 2,3-DHBA, azotochelin, and aminochelin.

[c] Ferric reductase activity expressed as nanomoles of Fe^{2+} formed per minute per milligram of protein.

[d] Cellular zinc levels determined by atomic absorption spectroscopy.

[e] N.A., not assayed.

obtained from an examination of the ferric reductase activity of cells grown with various minerals in the presence and absence of Zn^{2+} (data not shown). Only the extracts from cultures grown with the best iron sources, namely the minerals glauconite, olivine, and Fe_3O_4, exhibited decreased enzyme activity when 40 μM Zn^{2+} was also present. However, a determination of the ferric reductase activity in cultures grown with pyrite provided an anomalous result in that growth with Zn^{2+} did not cause an observable inhibition of activity. The reason for this is not known.

The ability of the ferric reductase to reduce the ferrisiderophores ferriazotobactin and ferriazotochelin was also examined. Although the use of the ferrisiderophores resulted in lower ferric reductase activity, the level of iron present during growth did not appear to affect the ability of the enzyme to reduce these iron chelates (Table 7). However, the presence of 40 μM Zn^{2+} during cell growth resulted in a significantly lower level of ferrisiderophore reduction than of ferric citrate reduction by the soluble cell extract (Table 7). The approximate decrease in reduction of the iron chelates by the Zn^{2+}-grown culture with respect to the Zn^{2+}-free culture was 25% for ferric citrate, 50% for ferriazotobactin, and 75% for ferriazotochelin.

Simultaneous examination of the ferric reductase activity of several samples was achieved through the use of nondenaturing polyacrylamide gels. The application of aliquots of soluble cell extracts to the gels followed by staining for enzyme activity revealed the presence of several bands of ferric reductase activity (Fig. 9). The general pattern of bands revealed by the activity stain consisted of a major spot in the centre region of the gel, with various faint bands present immediately above the main spot and also near the top of the gel. The protein profile of the most prominent minor bands was compared with that of the major spot by cutting the respective bands from the nondenaturing gel, boiling them in sample buffer containing SDS and ß-mercaptoethanol, and applying them to a denaturing polyacrylamide gel which was subsequently stained with Coomassie blue. The protein

TABLE 7. Effect of iron and zinc on ability of ferric reductase of soluble cell extracts to reduce various iron chelates

Growth conditions		Ferric reductase activity[a]			
		Ferric citrate		Ferri-azotobactin	Ferri-azotochelin
Fe (μM)	Zn (μM)	(30 μM)	(50 μM)	(30 μM)	(50 μM)
0.5	0	4.68	5.74	1.60	6.65
0.5	40	3.34	4.19	0.77	1.65
5	0	5.56	6.93	1.89	4.45

[a] Activity expressed as nanomoles of Fe^{2+} formed per minute per milligram of protein. All assays contained 0.3 mM NADH, 10 mM $MgCl_2$, 4 μM FMN, and 0.48 mM ferrozine.

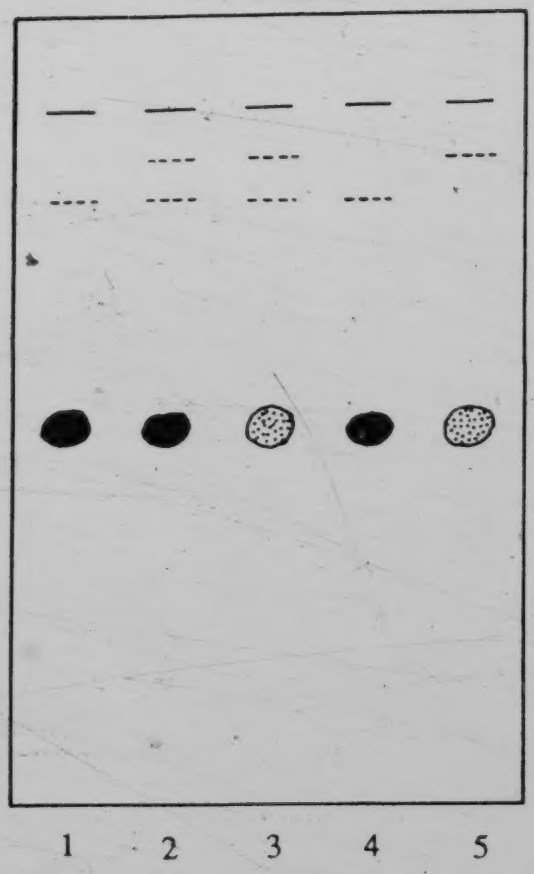

FIG. 9. Nondenaturing gel of ferric reductase activity. Samples analyzed include cytoplasmic extracts of cultures grown without any added iron or Zn^{2+} (lane 1), with 1 μM ferric citrate (lane 2), with 1 μM ferric citrate and 40 μM Zn^{2+} (lane 3), with 2.5 μM ferric citrate (lane 4), and with 2.5 μM ferric citrate and 40 μM Zn^{2+} (lane 5). The gel was stained to reveal ferric reductase activity as described in the Materials and Methods section. The solid dark spots indicate regions containing the most enzyme activity while the speckled spots and dashed lines indicate areas of minimal activity. A tracing of the gel is presented because the contrast between the purple bands revealed by the activity stain and the pink background was too low for successful photography.

profile from each of these samples was similar, suggesting that the minor bands of activity resulted from the formation of various protein aggregates rather than from different ferric reductases (data not shown). Growth of cultures with different levels of iron and Zn^{2+} did not change the pattern of bands revealed by the activity stain but did change the relative intensity of the bands: the bands from extracts of iron-sufficient cells grown in the presence of Zn^{2+} were much lighter than those from Zn^{2+}-free cultures under the same staining conditions (Fig. 9). As well, the addition of Zn^{2+} to the activity stain solution caused the band intensity of extracts of iron-sufficient cells to decrease.

The ferric reductase activity could also be inhibited by adding Zn^{2+} to the enzyme assays (Fig. 10). The addition of 10 μM Zn^{2+} to assays of cell extracts from cultures grown with 0.5 μM and 5 μM ferric citrate caused the ferric reductase activity to decrease by approximately 35%. The enzyme activity of the extract from the culture grown with 5 μM ferric citrate decreased steadily as increasing amounts of Zn^{2+} were added to the enzyme assay, culminating in an overall inhibition of 65% of the original activity. Further addition of Zn^{2+} to the extract from the culture grown with 0.5 μM ferric citrate had only a minimal effect on the ferric reductase activity (Fig. 10). The addition of increasing levels of Zn^{2+} to assays of the cell extract from a culture grown with 0.5 μM ferric citrate and 40 μM Zn^{2+} caused a general decrease in enzyme activity. The initial sharp drop in activity with added 10 μM Zn^{2+} that was observed with the other extracts was not seen in this case (Fig. 10). These results suggested that Zn^{2+} was unable to eliminate the ferric reductase activity completely.

The possibility that Zn^{2+} might be interfering with the enzyme assay rather than acting to inhibit the enzyme was also examined. The A_{562} values from enzyme-free ferric reductase assays containing known concentrations of Fe^{2+} (from a freshly made stock of $Fe(NH_4)_2(SO_4)_2$) with 0.16 mM and 0.8 mM ferrozine were determined. Rather than

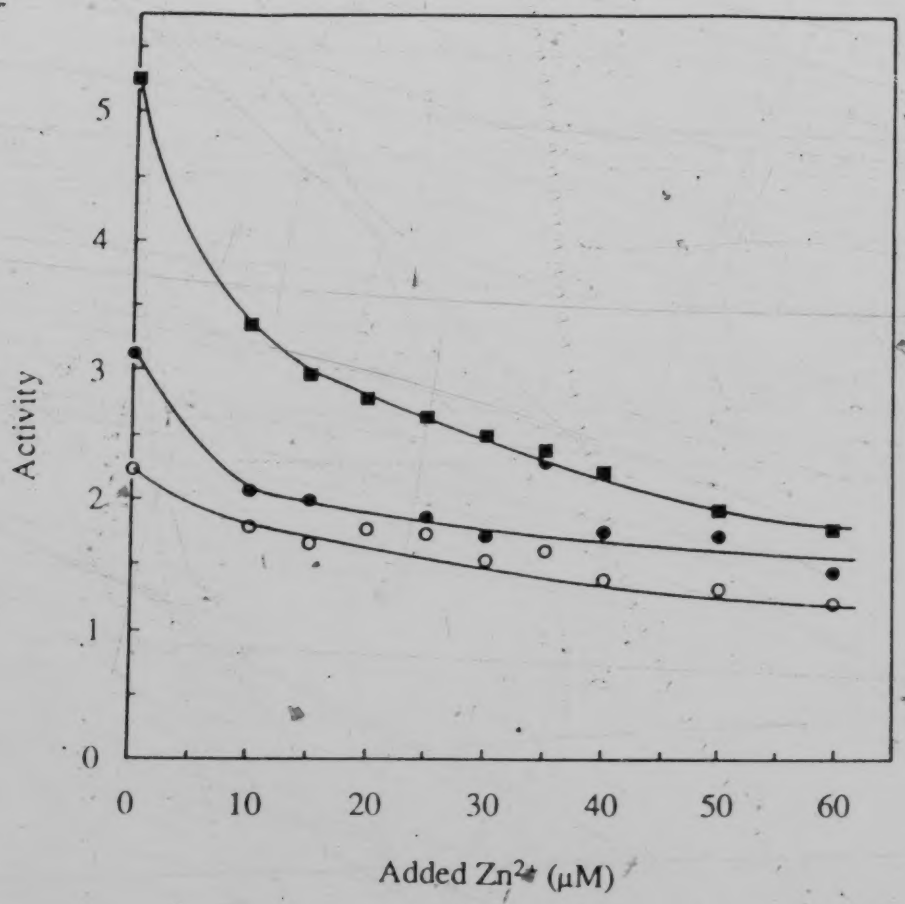

FIG. 10. Activity of the ferric reductase in the presence of increasing levels of Zn^{2+}. Samples tested were the soluble cell extracts from cultures grown in the presence of 5 μM ferric citrate (■), 0.5 μM ferric citrate (●), and 0.5 μM ferric citrate with 40 μM $ZnSO_4$ (○). Assay conditions were as described in the Materials and Methods section. Enzyme activity is expressed as nanomoles of Fe^{2+} formed per minute per milligram of protein.

deleteriously affecting the assay, the addition of Zn^{2+}, at levels ranging from 40 μM to 1 mM, appeared to cause a slight increase in the A_{562} (data not shown). In the assays containing 0.16 mM ferrozine, added 0.5 mM and 1 mM Zn^{2+} did cause a slight depression of the A_{562}; however, this effect was transient, with the A_{562} rapidly increasing to that obtained in the absence of any added Zn^{2+}. Of particular significance was the observation that the levels of Zn^{2+} which had been noted to affect the ferric reductase activity did not affect the formation of the Fe^{2+}-ferrozine complex.

The inhibitory effect of Zn^{2+} on the enzyme itself was confirmed by determining the activity of a partially purified enzyme preparation in the presence and absence of added 40 μM Zn^{2+} using both 0.16 mM and 0.8 mM ferrozine in the assay. The latter excess amount of ferrozine was used so as to overcome any potential competition between Zn^{2+} and Fe^{2+}. In each instance, the enzyme activity decreased by 40 to 50% in the presence of the Zn^{2+}.

Conditions for optimization of the assay for ferric reductase activity.

The ferric reductase activity of the soluble fraction of *A. vinelandii* was examined over the pH range 5.6 to 8.8 using the buffers 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 5.6, 6.0 and 6.8); 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 6.8, 7.2 and 7.6); and Tris-HCl (pH 7.6, 8.0 and 8.8). The optimum pH was found to be approximately 7.5 (Fig. 11).

A partially purified enzyme preparation obtained by passage of the soluble cell extract through a DEAE-Sephacel ion-exchange column was used to examine the effect of Mg^{2+} on the ferric reductase activity. When added to the ferric reductase assays, $MgCl_2$ stimulated enzyme activity up to a level that was more than sixfold higher than that obtained in its absence. The maximum level of activity was achieved with approximately 10 mM $MgCl_2$ (Fig. 12). The increased ferric reductase activity in the presence of Mg^{2+} led to an investi-

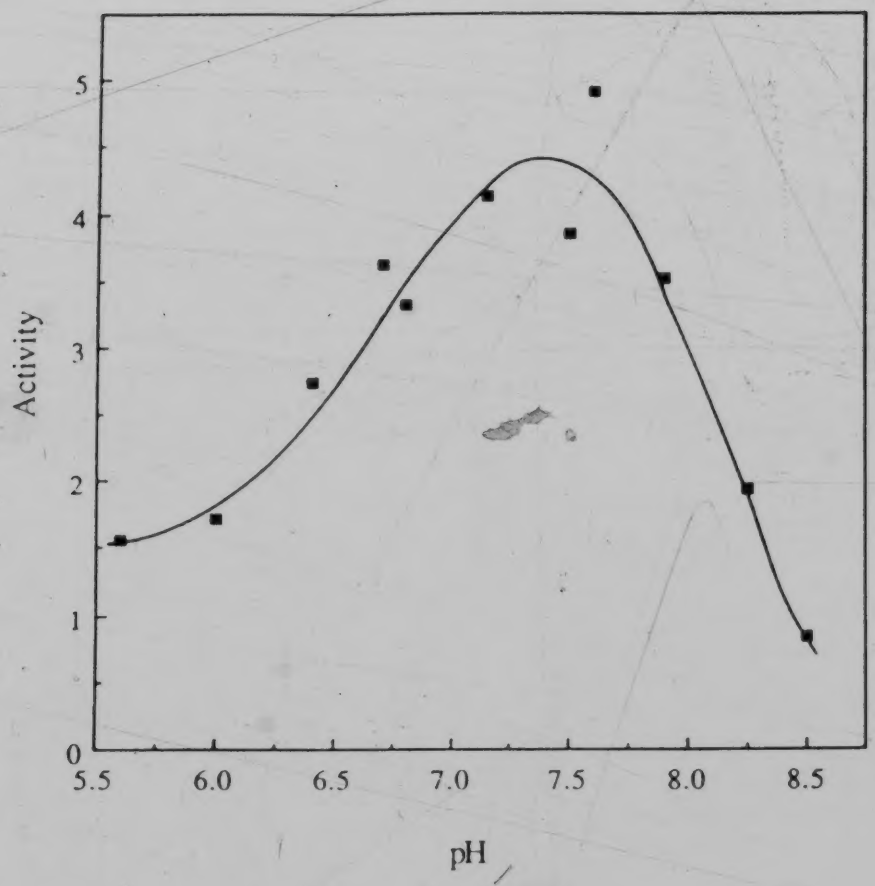

FIG. 11. Effect of pH on ferric reductase activity. Assay conditions were as described in the Materials and Methods section. Enzyme activity is expressed as nanomoles of Fe^{2+} formed per minute per milligram of protein.

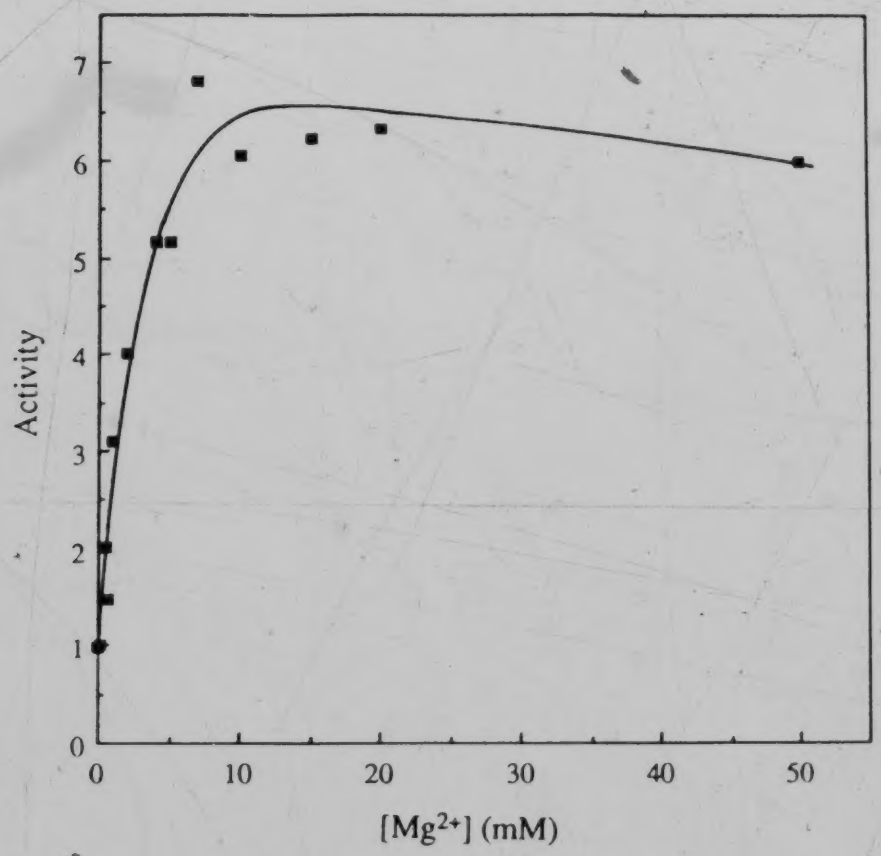

FIG. 12. Effect of Mg^{2+} on ferric reductase activity. Activity is expressed as nanomoles of Fe^{2+} formed per minute per milligram of protein. All assay conditions, except for the concentration of $MgCl_2$ added, were as described in the Materials and Methods section.

gation into the effect of two other group IIA ions, Ca^{2+} and Sr^{2+}, on the enzyme activity. When added at levels of 1 mM and 10 mM to ferric reductase assays, $CaCl_2$ stimulated the enzyme activity to the same extent as $MgCl_2$, while $SrCl_2$ was approximately half as effective as $MgCl_2$.

Determination of ferric reductase activity under partially anaerobic conditions was accomplished by sparging the assays with nitrogen gas for > 1 min prior to the addition of the enzyme solution. This typically resulted in up to a threefold increase in enzyme activity; however, the level of nonenzymatic reduction of iron also increased markedly. As well, widely varying levels of enzyme activity were obtained under these conditions. These problems made it difficul. to standardize the assay and led to the general use of aerobic conditions. An attempt to create a reducing environment by the addition of 10 mM ß-mercaptoethanol to the assay had no effect on enzyme activity while the presence of 1 mM dithiothreitol caused such a high rate of nonenzymatic iron reduction that accurate determination of the ferric reductase activity was impossible.

Location of ferric reductase activity.

In cells that had been broken either with a French pressure cell or by sonication, ferric reductase activity could be detected only in the soluble fraction. Although this suggested that the enzyme could be designated as being soluble, it did not provide an indication of whether the enzyme was cytoplasmic or periplasmic. There also was a possibility that the ferric reductase was loosely membrane-associated and that the harsh methods used to disrupt the cells dislodged the enzyme from its normal *in vivo* location.

To investigate this, cells were fractionated by several gentle methods and each of the resulting final fractions was assayed for ferric reductase activity (Table 8). The glycerol shock method, used in the preparation of extracts from *A. vinelandii* for the analysis of nitrogenase activity (Eady, 1980), furnished the complete soluble portion of the cells.

TABLE 8. Specific activities of ferric reductase and citrate synthase in various cell fractions[a]

Cell fraction	Specific activity of enzymes	
	Ferric reductase[b]	Citrate synthase[c]
Soluble (from glycerol shock)	4.15	1.40
Membrane	N.D.[d]	N.A.[e]
Lysozyme shock fluid	N.D.	0.23
Lysozyme "protoplasts"	4.27	1.81
Cold-osmotic shock fluid	0.80	1.22

[a] Assays performed on fractions from cells grown with 1 μM ferric citrate and no added Zn^{2+}.

[b] Activity expressed as nanomoles Fe^{2+} formed per minute per milligram of protein.

[c] Activity expressed as micromoles acetyl-CoA utilized per minute per milligram of protein.

[d] N.D., not detected.

[e] N.A., not assayed.

Sucrose gradients were used to isolate the membrane fraction from the material remaining after this gentle lytic treatment. Separation of the periplasm from the cytoplasm was attempted with two different methods, one involving cold-osmotic shock and the other utilizing lysozyme as a means of selectively releasing the outer membrane.

Ferric reductase activity was found in all fractions except those corresponding to membranes and those containing the "shock fluid" (presumably consisting of periplasmic components) from the lysozyme treatment (Table 8). Citrate synthase activity was used as an indication of the presence of cytoplasmic material; thus, the relatively high level of this activity in the cold-osmotic shock fluid compared to that in the lysozyme shock fluid suggested that the former procedure caused release of the total soluble fraction of the cells rather than just of the periplasmic components. Conversely, the low level of citrate synthase activity in the lysozyme shock fluid intimated that this fraction primarily contained periplasmic material; however, there was no periplasmic marker enzyme available to confirm this. Therefore, SDS-polyacrylamide gel electrophoresis was used in an effort to establish the identity of the fractions obtained from the different lysis procedures (Fig. 13). The dissimilarity in the pattern of the bands from the lysoyme shock fluid compared to that from the other cell fractions provided a further indication that the lysozyme treatment was successful in separating the cytoplasmic and periplasmic fractions of the cells. Thus, the ferric reductase appeared to be a cytoplasmic enzyme.

Purification of the ferric reductase.

Purification of the enzyme was originally attempted in the presence of the serine protease inhibitor PMSF. However, this compound was later found to have a considerable inhibitory effect on the enzyme activity, suggesting that the active site of the ferric reductase may contain a serine moiety. Therefore, the purification scheme was repeated in the absence of PMSF.

In each of the columns used in the purification of the enzyme, except the Phenyl

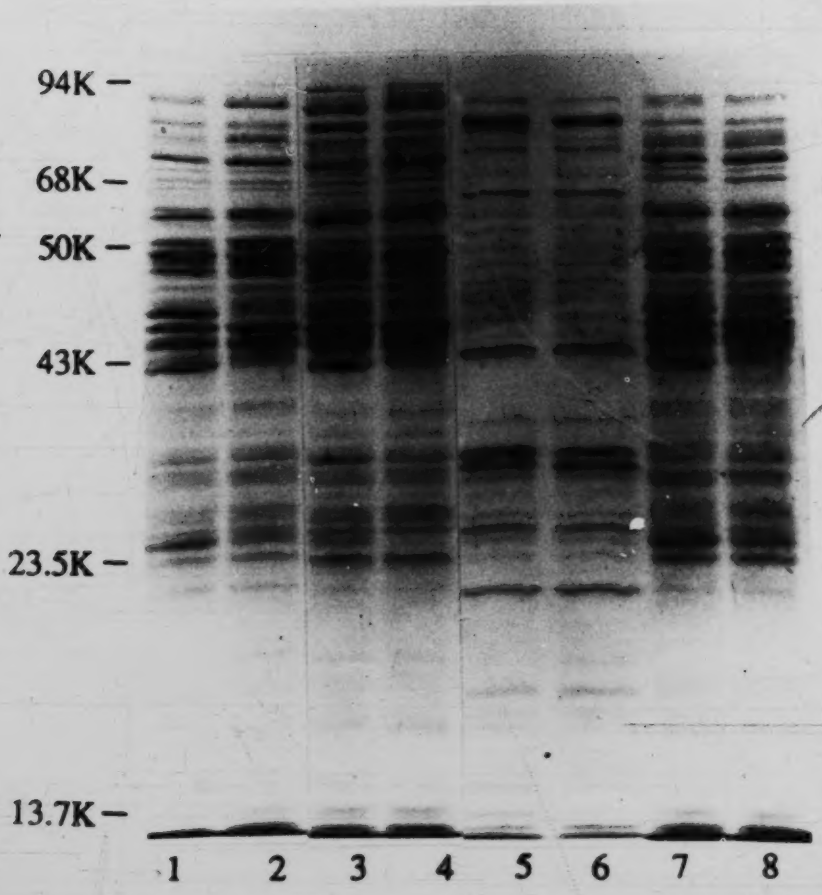

FIG. 13. SDS-polyacrylamide gel electrophoresis of the final fractions from the gentle cell lysis procedures. Lanes: 1 and 2, soluble fraction from glycerol shock; 3 and 4, cold-osmotic shock fluid; 5 and 6, lysozyme shock fluid; 7 and 8, lysozyme "protoplasts". Cultures were grown in the presence of 1 μM ferric citrate (lanes 1, 3, 5, and 7) and 2.5 μM ferric citrate (lanes 2, 4, 6, and 8).

Sepharose CL-4B column, all of the ferric reductase activity was found to elute as a single (if often broad) peak (Fig. 14, 15, 16, and 17). Evidence that a single enzyme was being purified was also obtained when aliquots of the concentrated peak regions from each of the columns were applied to a nondenaturing polyacrylamide gel and stained to reveal the ferric reductase: each fraction showed the enzyme activity in the same position (data not shown). Thus, the appearance of a broad peak of activity from the Phenyl Sepharose CL-4B column was felt to be a characteristic of a single ferric reductase rather than an indication of the presence of more than one enzyme. The pieces of the nondenaturing gel which contained ferric reductase activity were then boiled in sample buffer containing SDS plus ß-mercapto-ethanol and applied to a denaturing gel. Staining of this gel for protein provided confirmation that each column removed extraneous proteins (Fig. 18).

Determination of the specific activity of the enzyme in the various column fractions suggested that the enzyme had been purified 22-fold (Table 9). However, when a sample of enzyme from the Phenyl Sepharose CL-4B column was combined with a portion of the original soluble extract, the resulting level of ferric reductase activity was 2.7-fold greater than that obtained with the relatively pure enzyme alone. The addition of bovine serum albumin to the relatively pure enzyme did not cause the same dramatic increase in activity. Such a large increase in activity resulting from the addition of the crude soluble cell extract to the relatively purified enzyme suggested that perhaps some additional cofactor was lost during the purification procedure. Loss of enzyme activity due to degradation or as a result of storage did not appear to be a problem, since no decrease in activity was detected for samples at any stage of the purification procedure after long-term storage at -20°C or after repeated freeze-thaw cycles.

Determination of molecular weight of ferric reductase.

During the purification of the ferric reductase of *A. vinelandii*, the enzyme extract was loaded onto various gel filtration columns including Sephadex G-75 and G-100. However,

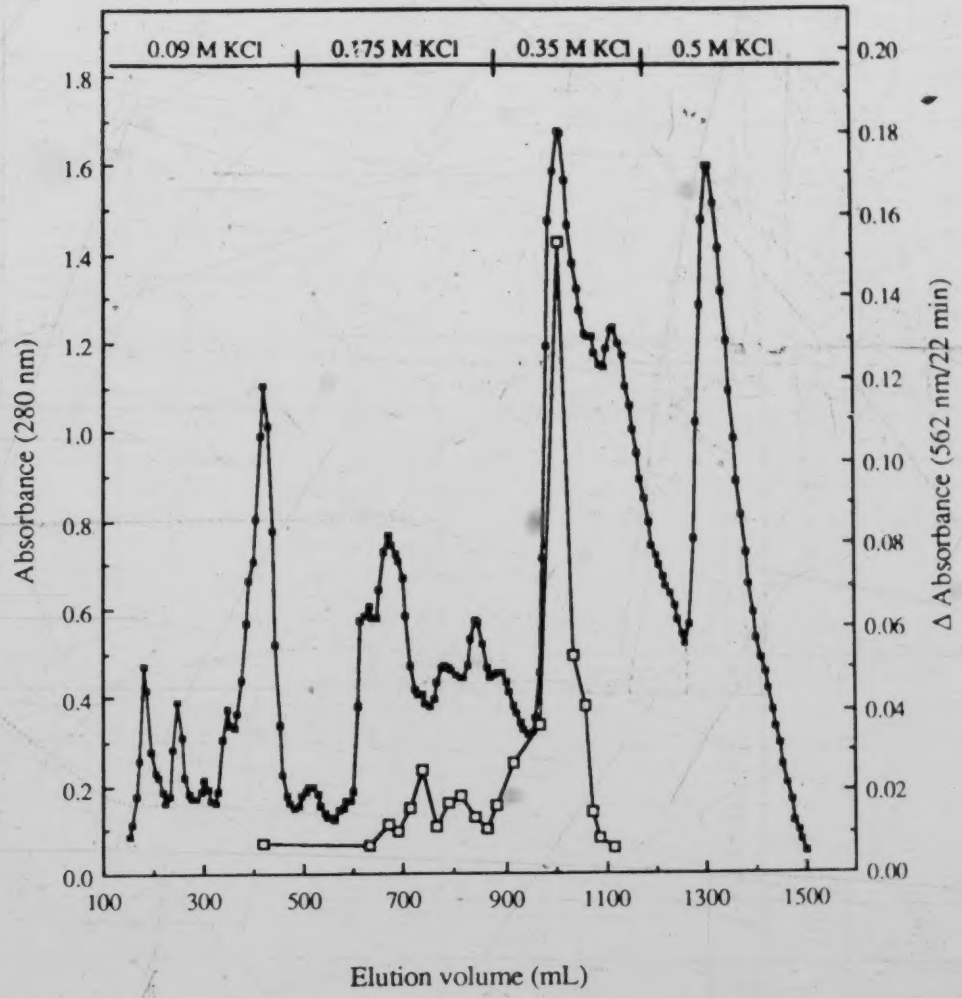

FIG. 14. First DEAE-Sephacel column of ferric reductase purification. Protein in column fractions was estimated by absorbance at 280 nm (■). Fraction volumes containing roughly equivalent amounts of protein were assayed for ferric reductase activity (□) under the conditions described in the Materials and Methods section. Enzyme activity is reported as the change in absorbance at 562 nm in 22 min. Concentrations of KCl used to elute the proteins are indicated at the top of the graph.

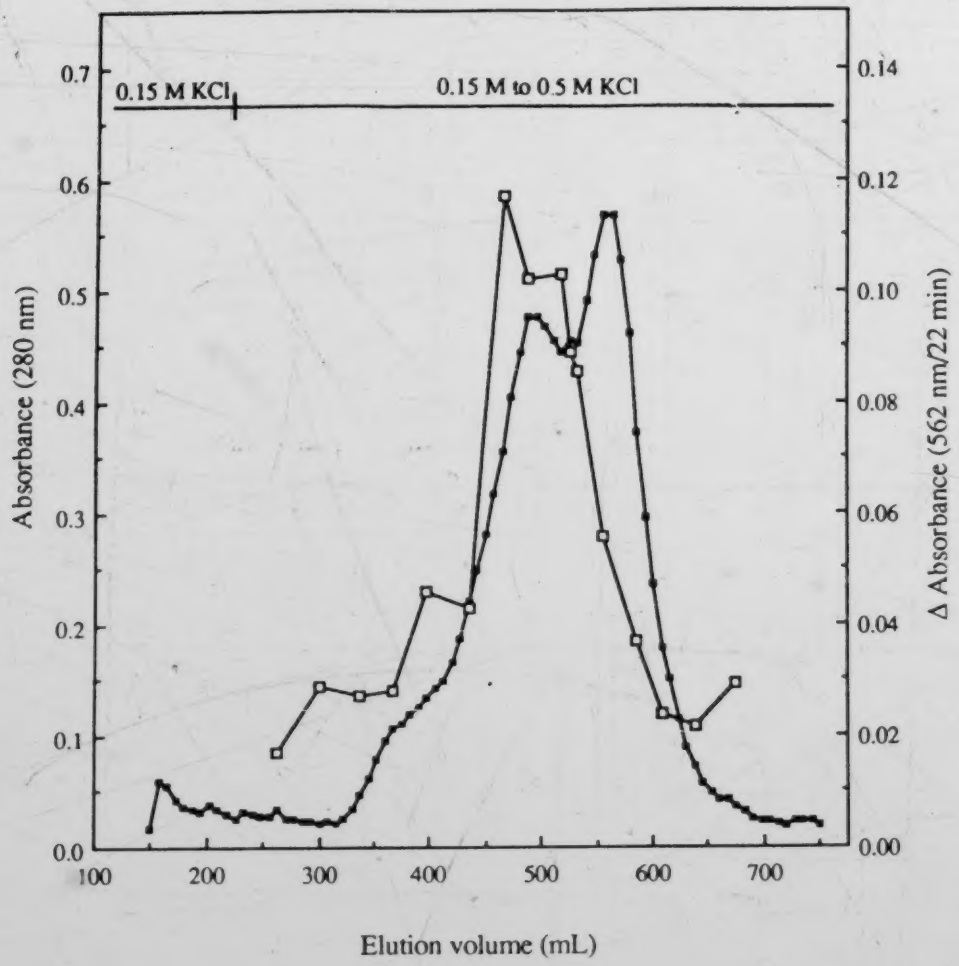

FIG. 15. Second DEAE-Sephacel column of ferric reductase purification. The sample applied to the column was eluted from the initial DEAE-Sephacel column. Protein in column fractions was estimated by absorbance at 280 nm (■). Fraction volumes containing roughly equivalent amounts of protein were assayed for ferric reductase activity (□) under the conditions described in the Materials and Methods section. Enzyme activity is reported as the change in absorbance at 562 nm in 22 min. Concentrations of KCl used to elute the proteins are indicated at the top of the graph.

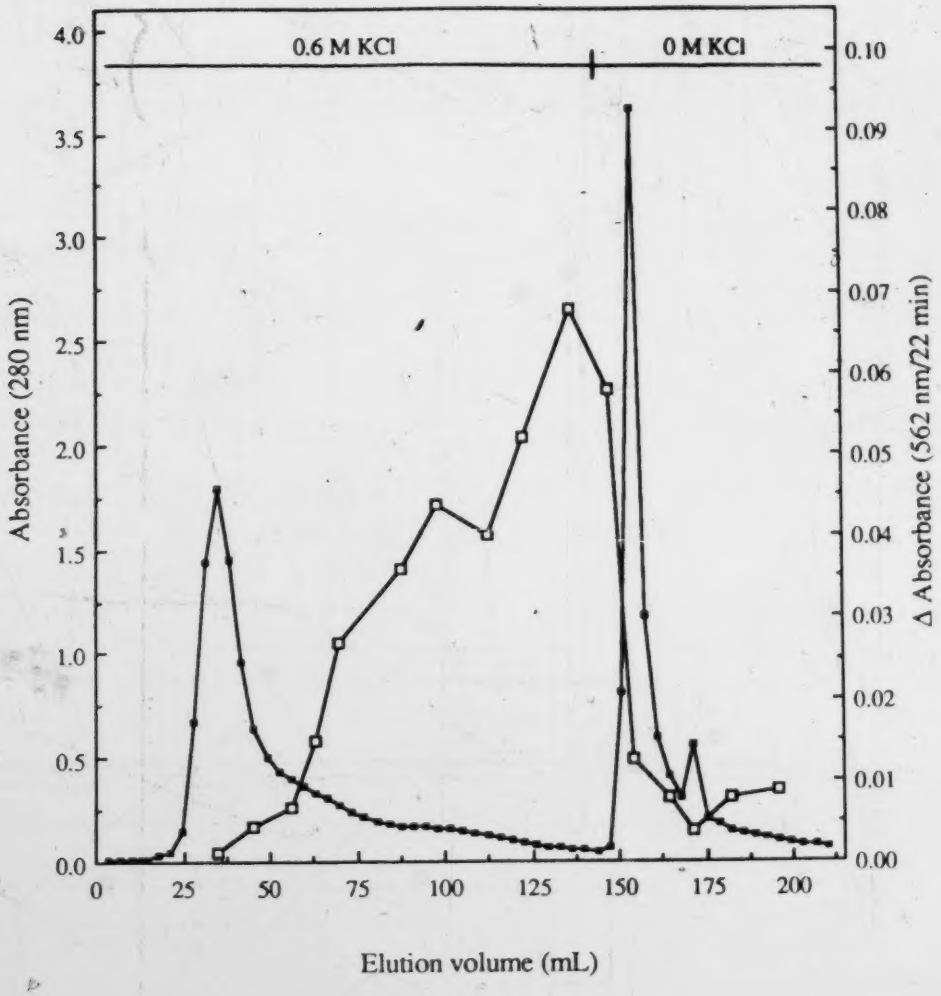

FIG. 16. Phenyl-Sepharose CL-4B column of ferric reductase purification. The sample applied to the column was eluted from the second DEAE-Sephacel column. Protein in column fractions was estimated by absorbance at 280 nm (■). Fraction volumes containing roughly equivalent amounts of protein were assayed for ferric reductase activity (□) under the conditions described in the Materials and Methods section. Enzyme activity is reported as the change in absorbance at 562 nm in 22 min. Concentrations of KCl used to elute the proteins are indicated at the top of the graph.

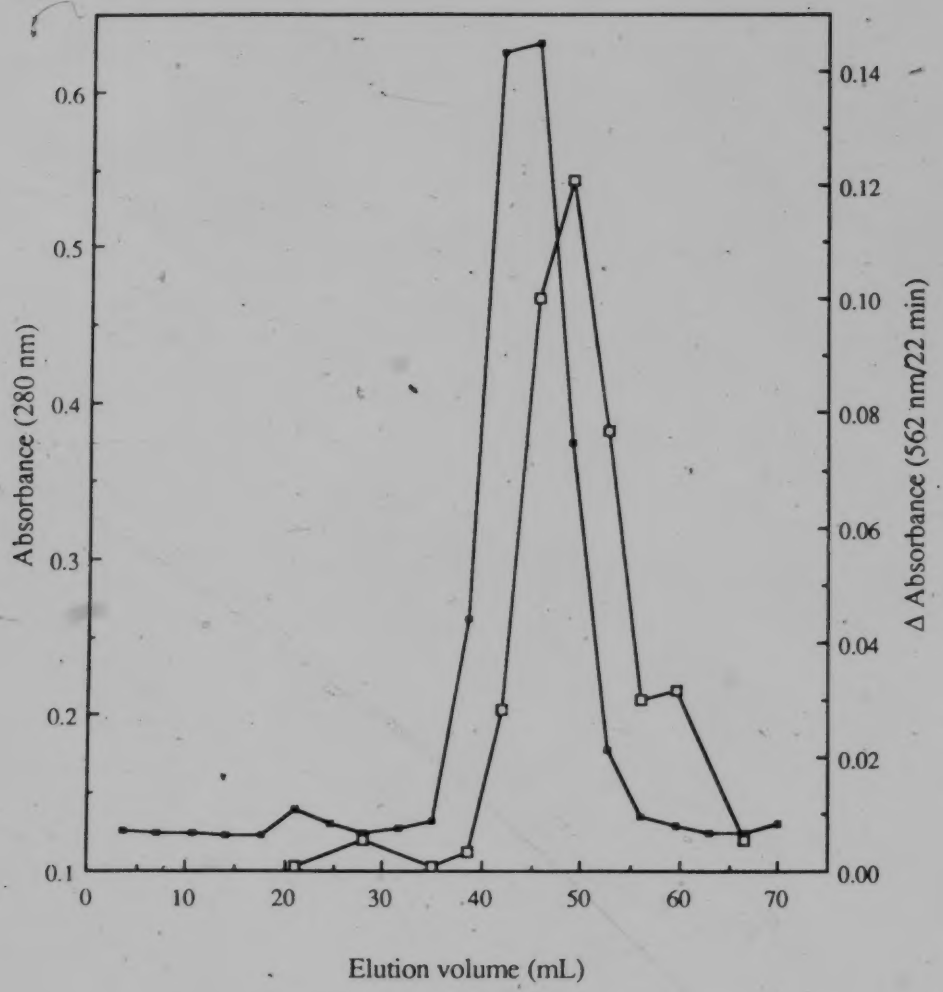

FIG. 17. Sepharose 4B column of ferric reductase purification. The sample applied to the column was eluted from the Phenyl Sepharose CL-4B column. Protein in column fractions was estimated by absorbance at 280 nm (■). Fraction volumes containing roughly equivalent amounts of protein were asayed for ferric reductase activity (□) under the conditions described in the Materials and Methods section. Enzyme activity is reported as the change in absorbance at 562 nm in 22 min.

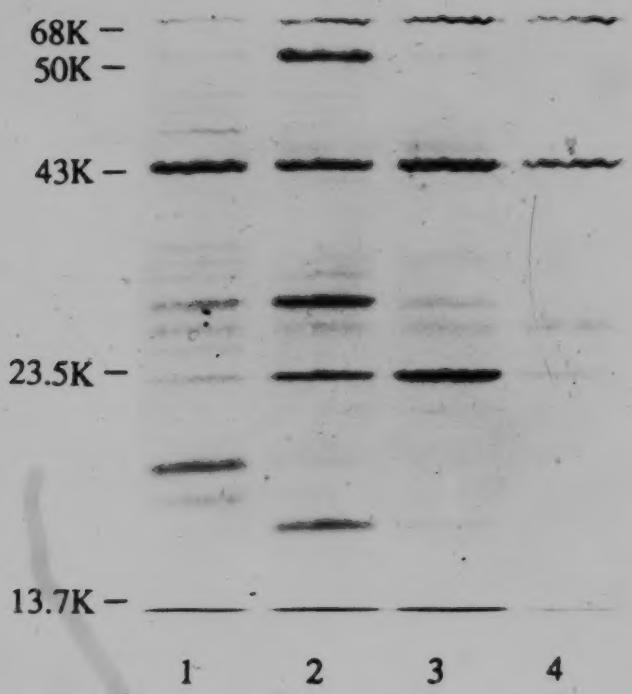

FIG. 18. SDS-polyacrylamide gel electrophoresis of the nondenaturing gel pieces which contained the major spot of ferric reductase activity. Samples analyzed were from the soluble cell extract (lane 1), and from the concentrated peak regions of the initial DEAE-Sephacel column (lane 2), the 2nd DEAE-Sephacel column (lane 3), and the Sepharose 4B column (lane 4).

TABLE 9. Purification scheme for ferric reductase

Fraction	Specific activity[a]	Purification (fold)	Relative amount of protein[b]
Soluble	11.1	1.0	100
1st DEAE-Sephacel	32.1	2.9	32
2nd DEAE-Sephacel	42.7	3.8	19
Phenyl Sepharose CL-4B	153	13.8	2.5
Sepharose 4B	244	22.0	0.75

a Specific activity expressed as nanomoles of Fe^{2+} formed per minute per milligram of protein. Each assay contained 0.4 mM NADH, 10 mM $MgCl_2$, 0.16 mM ferric citrate, 4 μM FMN, and 0.48 mM ferrozine. Reactions were initiated by the addition of the enzyme solutions.

b Refers to the total milligrams of protein present in each fraction and is presented as a percentage of the soluble cell extract.

determination of the molecular weight of the enzyme using these columns was not possible since the ferric reductase activity eluted in the void volume of the columns. Increasing the ionic strength of the column buffer with KCl ($\mu = 0.5$) did not change the elution pattern, suggesting that the molecular weight of the enzyme may be extremely large. The enzyme did not elute in the void volume of a Sepharose 4B column (fractionation range 6×10^4 to 20×10^6); however, the lack of suitable standards made it difficult to estimate the molecular weight of the enzyme.

Fractions from the Sepharose 4B column which exhibited enzyme activity were combined and concentrated, and a sample of this material, containing approximately 200 µg of protein, was applied to a nondenaturing acrylamide gel. After staining for ferric reductase activity, the spot which corresponded to the major region of activity was cut out of the gel, macerated, and boiled for 5 min in 60 µL of sample buffer containing SDS and ß-mercaptoethanol. This sample was then applied to an SDS-polyacrylamide gel which was made with a 2.5 cm stacking gel rather than the usual 1 cm one. Two major bands, with apparent molecular weights of 69,000 and 44,600, were revealed when this gel was stained for protein (Fig. 18).

Properties of the ferric reductase.

The apparent K_m values of the ferric reductase for Fe^{3+} (supplied as ferric citrate) and for NADH are 10 µM and 15.8 µM, respectively. The families of parallel lines obtained from plotting $1/v$ against either 1/[NADH] or $1/[Fe^{3+}]$ at increasing concentrations of the other substrate (Fig. 19 and 20), are consistent with a Ping Pong Bi Bi mechanism wherein a product is released between the addition of the substrates (Roberts, 1977).

The specificity of the ferric reductase with respect to several different substrates and cofactors was also examined. Enzyme activity in the presence of NADPH was 46% of that obtained with NADH, suggesting that NADH is the substrate of choice (Table 10). The

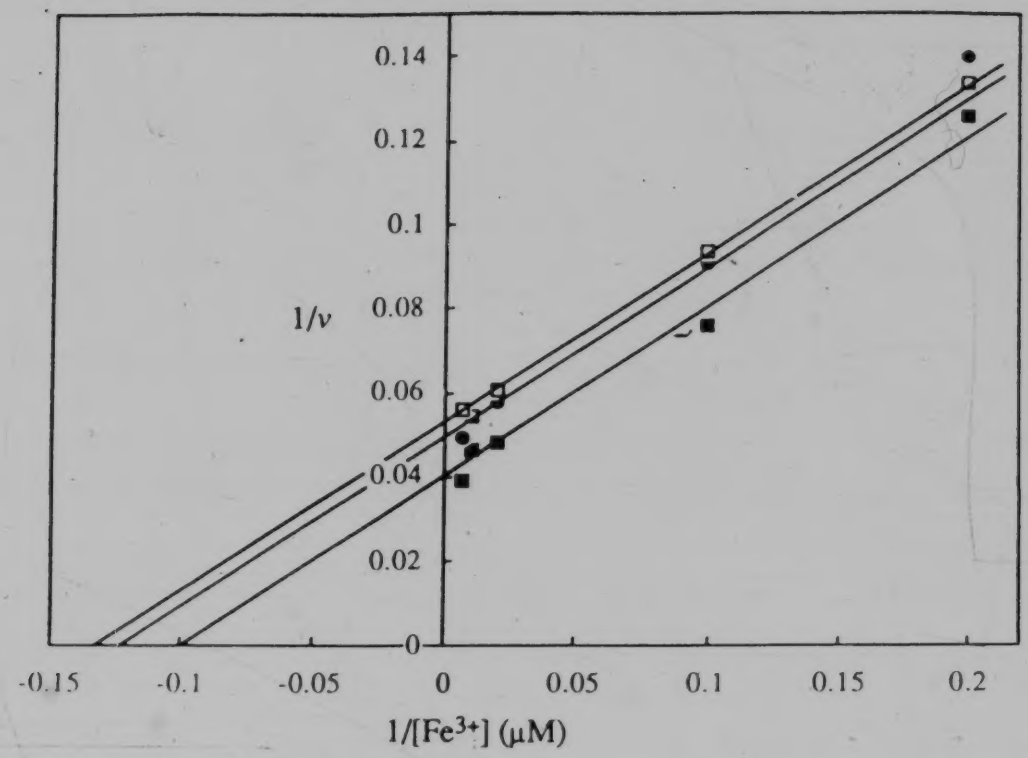

FIG. 19. Lineweaver-Burke plot of iron concentration and ferric reductase activity. $1/v$ is expressed as (nanomoles of Fe^{2+} formed per minute per milligram of protein)$^{-1}$. Each assay contained 0.48 mM ferrozine, 4 μM FMN, and 10 mM $MgCl_2$, with Fe^{3+} supplied as ferric citrate. NADH was present at concentrations of 60 μM (□), 120 μM (●), and 300 μM (■).

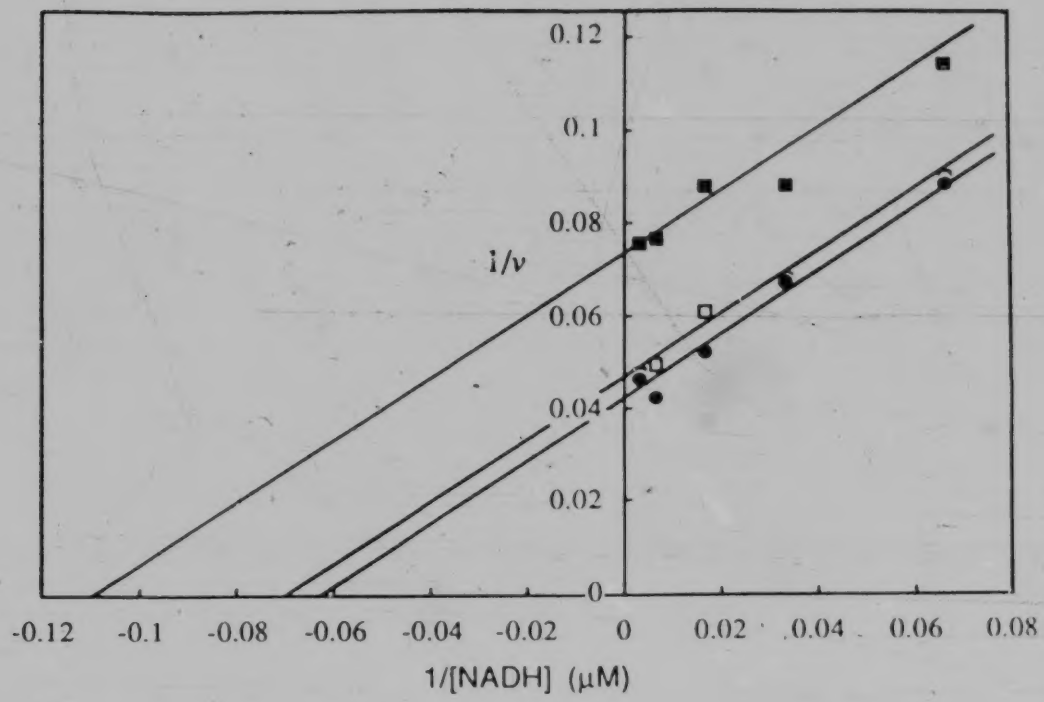

FIG. 20. Lineweaver-Burke plot of NADH concentration and ferric reductase activity. $1/v$ is expressed as (nanomoles of Fe^{2+} formed per minute per milligram of protein)$^{-1}$. Each assay contained 0.48 mM ferrozine, 4 μM FMN, and 10 mM $MgCl_2$. Ferric citrate was present at concentrations of 15 μM (■), 50 μM (□), and 100 μM (●).

TABLE 10. Effect of reductants and flavins on ferric reductase activity[a]

Substrate (Reductant)	Cofactor (Flavin)	Specific activity[b]
None	FMN	0
NADPH	FMN	11.5
NADH	FMN	25.1
NADH	None	1.65
NADH	FAD	3.02

[a] All assays contained 0.15 mM ferric citrate, 10 mM $MgCl_2$, and 0.48 mM ferrozine. When required, NADH and NADPH were added to a concentration of 0.3 mM and FMN and FAD were added to a concentration of 4 μM.

[b] Activity expressed as nanomoles of Fe^{2+} formed per minute per milligram of protein.

presence of a flavin was also necessary for maximum ferric reductase activity. FMN was preferred as a cofactor over FAD since the presence of 4 μM FMN stimulated the ferric reductase activity by a factor of 15, while an equal amount of FAD produced only a 1.8-fold increase in activity (Table 10). Although enzymatic activity during the purification procedure was based on ferric citrate reduction, the purified enzyme was able to reduce iron supplied as a number of different iron chelates (Table 11). This ability was seen with enzyme sampled from the end of the purification procedure as well as with partially purified enzyme from the beginning of the purification scheme (data not shown). The enzyme reduced iron bound to low-affinity chelates, such as citrate and 2,3-DHBA, more readily than iron bound to the high-affinity siderophores and to desferal (Table 11). However, the ability of the enzyme to reduce such diverse substrates suggested that the specificity of the enzyme for iron substrates was low.

Kinetic analysis of ferric reductase with added Zn^{2+} as an inhibitor indicated that Zn^{2+} affected both the V_{max} and the K_m values of the reaction (Fig. 21 and 22). Thus, Zn^{2+} was acting as a mixed-type inhibitor (Segel, 1975). The intersection points on the abscissas of the plots of $1/v$ *vs.* 1/[Fe] and $1/v$ *vs.* 1/[NADH] in the presence of different fixed concentrations of Zn^{2+} (Fig. 21 and 22) were used to calculate K_i values for Zn^{2+}, giving K_i of approximately 25 μM with respect to iron, and of approximately 1.7 μM with respect to NADH.

TABLE 11. Ability of the ferric reductase to reduce various iron chelates[a]

Iron chelate	Concentration (μM)	Specific activity[b]
Citrate	30	20.2
Citrate	50	21.0
Citrate	150	25.6
Azotobactin	30	1.02
Azotochelin	50	3.45
2,3-DHBA	150	15.9
Desferal[c]	150	5.63

[a] All assays contained 0.3 mM NADH, 10 mM $MgCl_2$, 4 μM FMN, and 0.48 mM ferrozine. The enzyme used to initiate the reactions was partially purified on a DEAE-Sephacel column.

[b] Activity expressed as nanomoles of Fe^{2+} formed per minute per milligram of protein.

[c] Desferal is the trade name for deferoxamine mesylate.

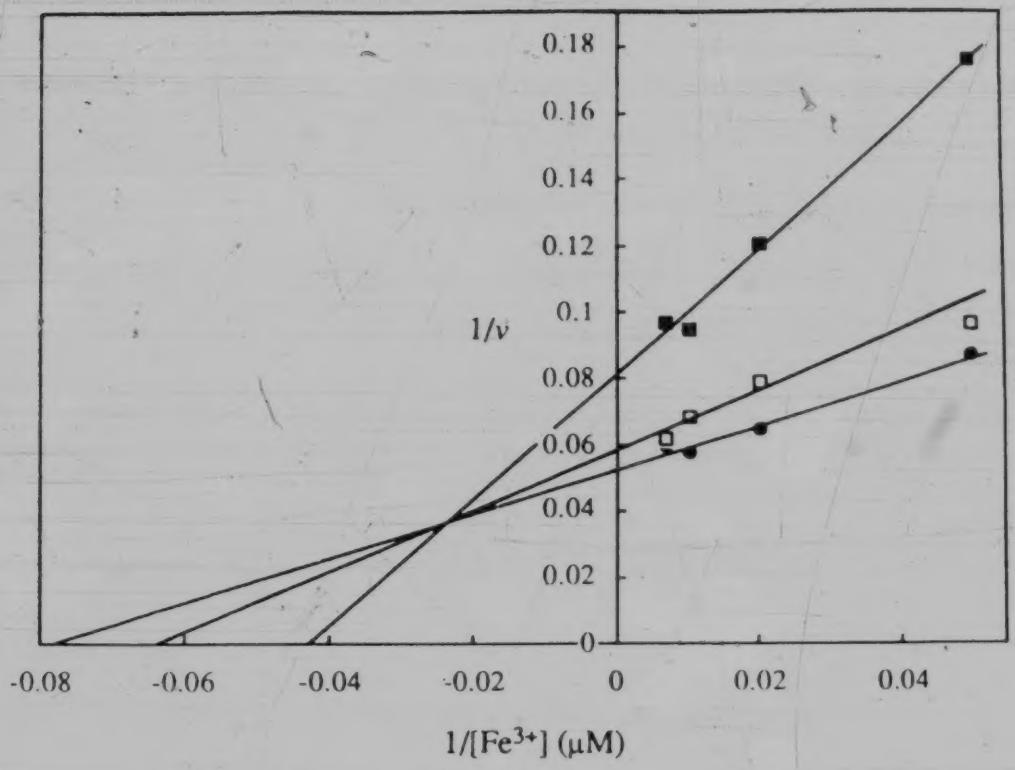

FIG. 21. Lineweaver-Burke plot of iron concentration and ferric reductase activity in the presence of the inhibitor Zn^{2+}. $1/v$ is expressed as (nanomoles of Fe^{2+} formed per minute per milligram of protein)$^{-1}$. Each assay contained 0.48 mM ferrozine, 4 μM FMN, 10 mM $MgCl_2$, and 0.3 mM NADH. $ZnSO_4$ was present at concentrations of 10 μM (●), 20 μM (□), and 100 μM (■).

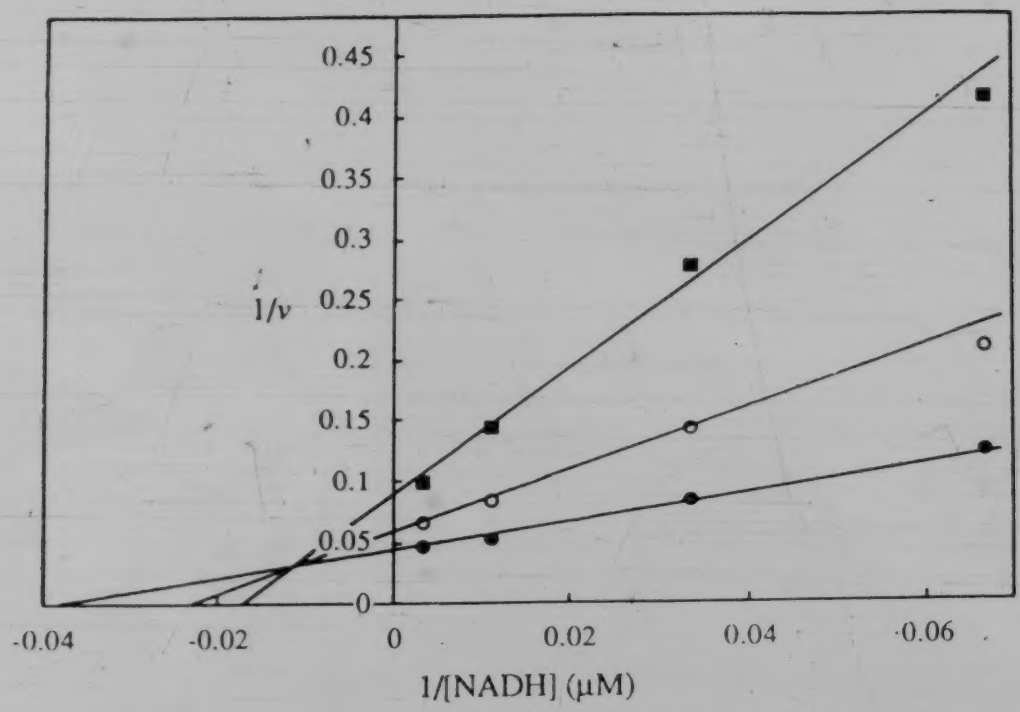

FIG. 22. Lineweaver-Burke plot of NADH concentration and ferric reductase activity in the presence of the inhibitor Zn^{2+}. $1/v$ is expressed as (nanomoles of Fe^{2+} formed per minute per milligram of protein)$^{-1}$. Each assay contained 0.48 mM ferrozine, 4 μM FMN, 10 mM $MgCl_2$, and 0.15 mM ferric citrate. $ZnSO_4$ was present at concentrations of 4 μM (●), 20 μM (○), and 100 μM (■).

DISCUSSION

The almost universal requirement of life forms for iron entails that the processes by which this metal is acquired by living organisms must be quite efficient as well as quite specific for iron. In most aerobic and facultatively anaerobic microorganisms, the high-affinity procurement of iron from the environment is accomplished through a three-stage operation: first, the synthesis of siderophores to chelate and solubilize iron; second, the binding of the ferrisiderophores to outer membrane receptor proteins in order to achieve internalization of the ferric chelate; and finally, an intracellular enzymatic process whereby the iron is released to a variety of iron-requiring enzymes and proteins (Crichton and Charloteaux-Wauters, 1987). The principal regulator of the complete system is iron; however, this study has indicated that Zn^{2+}, at least in *Azotobacter vinelandii*, can also have an effect.

The presence of Zn^{2+} during growth of iron-limited cultures of *A. vinelandii* caused early initiation of the rapid phase of siderophore production (particularly of the catechol-type siderophores) as well as overproduction of these compounds. However, as the levels of Zn^{2+} required to obtain these results did not appear to deleteriously affect cell growth, this indicated that the increased siderophore production was not simply a consequence of massive metabolic stress. As well, Zn^{2+} did not affect the total cellular iron content of the cells, thus suggesting that Zn^{2+} was not inducing a condition either of more severe or of earlier iron-limitation.

The lack of a direct effect of Zn^{2+} on the components of the first stage of the process of iron acquisition (*i.e.*, the siderophores) was demonstrated by the inability of Zn^{2+} to compete successfully with iron for binding to the siderophores of *A. vinelandii* (Table 2). However, Zn^{2+} may have indirectly affected the siderophores by interfering in the regulation of transcription of the siderophore biosynthesis genes, since azotobactin production was most dramatically affected as a result of cell growth in the presence of Zn^{2+}. Produc-

tion of azotobactin is tightly controlled by the level of available iron and is repressed at a much lower iron concentration than that required to repress synthesis of azotochelin and aminochelin (Page and Huyer, 1984). This suggests that the regulation of azotobactin synthesis may be separate from the regulation of production of the catechol-type siderophores (Page and von Tigerstrom, 1988). A molecular mechanism for iron assimilation has been proposed for *E. coli*, where the *fur* gene product has been identified as a negative regulator of expression of siderophore-mediated, high-affinity iron transport systems (Bagg and Neilands, 1985; Hantke, 1981). Fur protein binding to iron-regulated promoter sequences is primarily achieved through the formation of an Fe^{2+}:Fur repressor complex, although certain other divalent heavy metals are capable of interacting with the Fur protein. When tested alone, Zn^{2+} was found to be only partially effective. However, when combined with Mn^{2+} (which is an effective substitute for Fe^{2+} in activating the Fur protein), Zn^{2+} decreased the ability of Mn^{2+} to activate the Fur protein (de Lorenzo *et al.*, 1987). Although there has not, as yet, been a description of a parallel regulatory system in *A. vinelandii*, the existence of such a system could explain the effect of Zn^{2+} on the biosynthesis of the siderophores in general and on azotobactin in particular. Separate regulation of azotobactin and the catechols could result in azotobactin synthesis being more strictly regulated by a Fur:Fe^{2+} complex than the catechols. Thus, Zn^{2+} may have a more disruptive effect on Fe^{2+} repression of the azotobactin system than the catechol siderophore system. However, while there is no evidence to prove or disprove the existence of such a system in *A. vinelandii*, interference in the regulation of the siderophore biosynthesis genes would not be expected to cause all the observed differences between Zn^{2+}-grown and Zn^{2+}-free cells.

Overproduction of the siderophores may also have been indirectly due to Zn^{2+}-mediated repression of production of the ferrisiderophore receptors. A decrease in the quantity of these proteins would diminish the transport efficiency of the iron complexes into the cell.

However, normal production in the presence of Zn^{2+} of the iron-repressible outer membrane proteins which are postulated to function as ferrisiderophore receptors (Page and von Tigerstrom, 1982; Page and Huyer, 1984; Page and Grant, 1988), led to a rejection of this possibility (Fig. 6A).

As a consequence of their ability to wrest iron from a variety of substances including minerals, the affinity that siderophores have for iron, and specifically for Fe^{3+}, is very high (Neilands, 1981b). Release of the iron from the ferrisiderophores is accomplished through processes which reduce Fe^{3+} to Fe^{2+} and lead to the subsequent release of the iron to a variety of cellular components (Crichton and Charloteaux-Wauters, 1987). Therefore, interference in this process would presumably decrease the amount of Fe^{2+} within the cell. Inasmuch as Fe^{2+}, in a free, intracellular pool, has been proposed to be the major controlling factor for iron uptake (Williams, 1982), lower levels of this ion could cause the observed increase in siderophore synthesis. An investigation into the relative levels of Fe^{3+} and Fe^{2+} in *A. vinelandii* cells grown in the presence and absence of Zn^{2+} confirmed the existence of a relationship between the presence of Zn^{2+} and decreased levels of Fe^{2+} (Tables 5 and 6). These results suggested that the process of reducing Fe^{3+} to Fe^{2+} was hindered by the presence of Zn^{2+}.

In most organisms, the siderophores are transported into the cell as the complete ferrisiderophore complexes for reductive removal of the iron. Reduction, therefore, is presumably not directly involved in the uptake process (Emery, 1987). However, if the process of dissociation of iron from the ferrisiderophores was affected, continued uptake of the ferrisiderophore complexes would result in a sharply increased level of these compounds within the cell. Since the intracellular ferrisiderophore pool size is presumably finite, a consequence of an accumulation of ferrisiderophores could be a feedback inhibition-type situation which would impact detrimentally on the process of iron uptake. Therefore, the release of iron from the ferrisiderophores to the cells may be considered to be only one of a

series of steps in the process of iron uptake, and interference in this step could affect the entire iron uptake system. The presence of Zn^{2+}, either in the uptake solutions or in the growth medium, caused a decrease in the rate of both high- and low-affinity iron uptake by *A. vinelandii* (Table 3). Since Zn^{2+} was not in direct competition with iron for uptake, this suggested that Zn^{2+} was affecting some site that was common to both uptake processes. Thus, as *A. vinelandii* is supplied with iron through the actions of three different siderophores as well as by the low-affinity iron uptake system (Knosp *et al.*, 1984; Page and Huyer, 1984; Page and von Tigerstrom, 1988), an enzyme capable of reducing Fe^{3+} bound to a variety of chelates (*i.e.*, a ferric reductase) was a likely candidate for being susceptible to inhibition by Zn^{2+}.

A ferric reductase was identified in crude, soluble cell extracts of *A. vinelandii*. The enzyme was active under aerobic conditions; however, in common with most ferric reductases from other microorganisms (Arceneaux, 1983), the creation of a partially anaerobic environment did result in increased activity. The activity of the enyzme was not affected by the iron concentration of the growth medium; therefore, the enzyme appeared to be constitutive. A lack of any relation between the iron status of the cells and the activity of ferric reductases is a typical characteristic. So far, only the ferric reductase from *Neurospora crassa* has been distinguished as an exception to this general rule (Ernst and Winkelmann, 1977). Comparison of the ferric reductase activity in cellular fractions of *A. vinelandii* designated the cytoplasm as the location for the enzyme. Although most ferric reductases, including those from *Rhodopseudomonas sphaeroides* (Moody and Dailey, 1985) and *Bacillus megaterium* (Arceneaux and Byers, 1980), are cytoplasmic, this is by no means a universal trait, since the iron reductase activity in *Spirillum itersonii* is associated with the respiratory chain (Dailey and Lascelles, 1977) and the ferripyochelin iron reductase of *Pseudomonas aeruginosa* is a periplasmic enzyme (Cox, 1980).

Estimation of the molecular weight of the ferric reductase was difficult due to a lack of correlation between the results provided by gel filtration chromatography and SDS-polyac-

rylamide gel electrophoresis. The former method suggested that the enzyme may be extremely large, while two major proteins of apparent molecular weights of 69,000 and 44,600 were revealed by the latter method. SDS-polyacrylamide gel electrophoresis thus suggested that the enzyme could be composed of two different subunits (or multiples thereof) or of a single sized subunit. In the latter instance one of the two proteins would be a contaminant. Consequently, the enzyme may be similar to that of *R. sphaeroides* which has been proposed to be a single subunit protein with a molecular weight of 32,000 (Moody and Dailey, 1985). The molecular weights of other ferric reductases have not been determined. Alternatively, there is a possibility that the ferric reductase activity from *A. vinelandii* is part of an extremely large enzyme complex, analogous to that of *B. subtilis* wherein the ferrisiderophore reductase activity is associated with an aromatic biosynthetic enzyme complex (Gaines *et al.*, 1981). However, the observed migration of the active enzyme halfway down a nondenaturing gel (Fig. 9) is not typical of an extremely large protein. In addition, the pattern of protein elution from the Sepharose 4B column in which the ferric reductase activity was associated with the second half of a single large peak (Fig. 17) implied that the high molecular weight value suggested by this method was an artefact. It is possible that during purification the enzyme underwent an association process similar to that obtained during purification of a pyridine-nucleotide transhydrogenase from *A. vinelandii* (van den Broek *et al.*, 1971, 1971b). A complex formed by association might not separate during gel filtration chromatography but would be dissociated by the procedure used in the preparation of the sample for loading on an SDS-polyacrylamide gel. Further purification of the active enzyme, perhaps using high performance liquid chromatography (HPLC), is necessary to determine the actual molecular weight of the ferric reductase.

The properties of the enzyme were similar to those recorded for other ferric reductases in that it utilized NADH as a reductant, required FMN as a cofactor, and was stimulated by the presence of a divalent group IIA cation. Kinetic analysis of the dependence of the K_m values for NADH and iron on the concentration of the other substrate revealed a pattern

typical of a Ping Pong Bi Bi type of reaction mechanism (Segel, 1975). During enzymatic reactions of this kind, a product is released before all the substrates have been added; thus, the enzyme oscillates between two different stable forms. Therefore, as NADH was presumably used by the ferric reductase as a source of electrons for the reduction of iron, the enzyme probably bound this substrate first. The Fe^{3+}-chelate would then not be bound until after the enzyme released NAD^+. This mechanism is quite different from that of *R. sphaeroides* (Moody and Dailey, 1985), which is the only other iron reductase for which a reaction mechanism has been suggested and which apparently requires that both substrates be bound before products are released. This is typical of either an Ordered Bi Bi reaction mechanism wherein the order in which the substrates are bound and the products released is fixed, or of a Rapid Equilibrium Random Bi Bi mechanism in which the order of substrate binding and product release is random (Segel, 1975). The first mechanism was proposed as that used by the iron reductase of *R. sphaeroides* (Moody and Daily, 1985); however, no evidence was presented to indicate that product inhibition studies, which are necessary to distinguish between the two mechanisms (Segel, 1975), had been done.

The pattern of enzyme inhibition resulting from the addition of Zn^{2+} to the ferric reductase of *A. vinelandii* was characteristic of a mixed-type inhibition system (Segel, 1975). This designation was refined by replotting the primary reciprocal plot data as slope *vs.* Zn^{2+} concentration and $1/v$-axis intercept *vs.* Zn^{2+} concentration (data not shown). These replots were hyperbolic which indicated that Zn^{2+} was a hyperbolic (partial) mixed-type inhibitor. In this type of system, which is a mixture of partial competitive and partial noncompetitive inhibition, both the enzyme-substrate complex and the enzyme-substrate-inhibitor complex will form product, but the rates at which they do so differ (Segel, 1975). Therefore, the enzyme activity could not be completely inhibited by Zn^{2+}, a result that was also suggested by the observation that the ferric reductase activity in soluble cell extracts decreased hyperbolically with increasing amounts of Zn^{2+} (Fig. 10).

A survey of the effect of various ions, including Zn^{2+}, on ferric reductase activity has been carried out in a few organisms including *B. subtilis* (Gaines *et al.*, 1981), *N. crassa* (Ernst and Winkelmann, 1977), and *R. sphaeroides* (Moody and Dailey, 1983). In each of these systems, Zn^{2+} was not observed to inhibit the ferric reductase; therefore, the inhibitory action of Zn^{2+} on this enzyme may not be ubiquitous. However, it is possible that Zn^{2+} may affect the ferric reductase of *Pseudomonas fluorescens* since the presence of this ion resulted in increased siderophore production (Baghdiantz, 1952; Chakrabarty and Roy, 1964).

The ferric reductase of *A. vinelandii* was able to use as a substrate a variety of iron-chelates, including the siderophores ferriazotobactin and ferriazotochelin. The relative level of reduction of the ferrisiderophores compared to ferric citrate was higher in assays containing crude soluble cell extract than when the purified enzyme was used (Tables 7 and 11). These results suggested that some factor present in the soluble cell extract, but lost during the purification procedure, was necessary for optimal levels of reduction of all possible iron chelates, from ferric citrate to the ferrisiderophores. Maximal enzyme activity may require the presence of another cofactor or may only be possible under anaerobic conditions or in a strict reducing environment. Although neither of these possibilities can be ruled out, no ferric reductases thus far described have been demonstrated to require an additional cofactor. However, detectable levels of ferripyoverdine reduction by the ferrisiderophore reductase activity in extracts of *P. fluorescens* were obtained only under strictly anaerobic conditions (Meyer *et al.*, 1987).

A potential physiological role for the ferric reductase is the reduction of a variety of ferric chelates. However, as it is able to reduce the ferrisiderophores, the primary role of the enzyme in iron-limited cells is most likely the dissociation of iron from the siderophores. The location of the ferric reductase within the cytoplasm therefore necessitates transport of the ferrisiderophore complexes into the cell. No studies have been done to determine the fate of the *A. vinelandii* ferrisiderophore complexes; however, it has been

speculated that pyoverdine, which is similar in structure to azotobactin (Demange *et al.*, 1986, 1987), is hydrophobic enough on its outer surface to allow transport through the cell membranes (Wendenbaum *et al.*, 1983). The proposed function of the *A. vinelandii* enzyme is similar to that put forth by Arceneaux (1983) as a general role for ferric reductases, but differs from that of the iron reductase of *R. sphaeroides* which was capable of reducing iron supplied as an iron-Tris chelate but not as the ferrisiderophores (Moody and Dailey, 1985). The primary role for this enzyme was suggested to be that of providing Fe^{2+} to ferrochelatase for use in heme synthesis and possibly also that of controlling the flow of electrons within the cell.

A physiological role of reducing a variety of ferric chelates could suggest that the ferric reductase is a common point in the general pathways of both high- and low-affinity iron uptake. However, the ability of Zn^{2+} to inhibit the activity of the ferric reductase does not explain the observation that the presence of Zn^{2+} in the uptake medium decreased azotobactin-mediated ^{55}Fe uptake markedly in Zn^{2+}-free cells but only slightly in Zn^{2+}-grown cells (Table 3). One possible explanation for this is that the extreme iron-limited conditions which led to derepression of azotobactin synthesis may also have caused production of another ferric reductase which specifically reduced ferriazotobactin and was possibly less sensitive to inhibition by Zn^{2+}. Although most ferric reductases thus far described show activity towards a number of different iron sources (Arceneaux and Byers, 1980; Gaines *et al.*, 1981; Lodge *et al.*, 1982; McCready and Ratledge, 1979; Straka and Emery, 1979), this is not a general attribute since a reductase specific for ferripyochelin reduction has been described in *P. aeruginosa* (Cox, 1980). The existence of an enzyme able to reduce ferriazotobactin but not ferric citrate would not have been realized during the purification procedure since the criterion for purification was the ability to reduce ferric citrate. However, the soluble cell extract from a culture grown with a level of iron sufficient to depress azotobactin synthesis exhibited the same level of ferriazotobactin reductase activity as the soluble cell extract of a culture grown with a low level of iron (Table 7). If *A. vinelandii* produced

a reductase that was active only towards ferriazotobactin, then production of this enzyme would presumably accompany the synthesis of azotobactin; therefore, the specific activity of ferriazotobactin reduction should be higher in extracts of cultures that synthesized azotobactin compared to that in extracts of iron-sufficient cultures.

An alternate interpretation for the minimal effect of Zn^{2+} on azotobacin-mediated ^{55}Fe uptake by Zn^{2+}-grown cells would be the existence of an enzyme whose function is to destroy the ferriazotobactin complex. By being separate from the ferric reductase, the enzyme would thus augment the process of iron release from this ferrisiderophore. Such an enzyme would presumably be synthesized coordinately with azotobactin; therefore, although the presence of Zn^{2+} during cell growth would not necessarily have a direct effect on the synthesis of this enzyme, Zn^{2+} could indirectly affect its production by increasing the levels of azotobactin. Although the ferric reductase was able to reduce ferriazotobactin, the specific activity of ferric citrate reduction (as determined under aerobic assay conditions) was much higher. The apparent dislike of the enzyme for ferriazotobactin is difficult to reconcile with the fact that azotobactin is the most efficient of the three siderophores in transporting iron into the cell (Page and von Tigerstrom, 1988). Therefore, destruction of the ferrisiderophore, possibly resulting in the formation of a cleavage product with a redox potential within the range of physiological reductants such as NADH, may be necessary to compensate for the low reduction activity with respect to the intact molecule. Thus, this could obviate the need for the reductase. Enzymatic destruction of ferric enterobactin is accomplished through the actions of an esterase which hydrolzes the ester bonds between the dihydroxybenzoylserine moieties of enterobactin and yields a ligand which has much lower affinity for Fe^{3+} than the intact siderophore (O'Brien *et al.*, 1971). However, as azotobactin is a fluorescent peptide (Demange *et al.*, 1986), enzymatic destruction of this compound would be accomplished best through the actions of a protease. Although the existence of such a protease was not examined in this study, high levels of proteolytic enzymes have been observed under conditions of iron-deficiency in *A. vinelandii*

(Fukasawa *et al.*, 1976).

The processes by which living organisms assimilate iron are, by virtue of the extreme insolubility of this metal ion in neutral, aerobic environments, quite efficient but also quite complex. The first suggestion that microorganisms may produce siderophores in order to make inorganic iron available for assimilation occurred over 30 years ago (Neilands, 1957) and since then much information has been accumulated regarding the structures of these iron-chelating compounds (Neilands, 1981b). However, only recently has a model for regulation of iron uptake been presented (Bagg and Neilands, 1987), and the exact processes by which siderophore-bound iron is donated to cellular components are still under investigation (Emery, 1987). Although this study does not provide an unequivocal explanation of the process of iron assimilation in general, or even in *A. vinelandii* in particular, the discovery that Zn^{2+} inhibited the ferric reductase of this organism and the apparent consequences of this inhibition on other aspects of iron assimilation, suggests an interrelatedness of all components of the system for the procurement of iron in *A. vinelandii*. There are still many questions regarding the manner in which iron is acquired by microorganisms, including amongst them a definition of the exact role of ferric reductases. However, while this research does not provide all the answers, the results and theories present herein will hopefully assist in the elucidation of the complete process of iron assimilation.

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